Retinal Progenitor Cell Lines

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For my parents

Thank you for everything

Abstract

Inherited retinal dystrophies and age-related macular degeneration (AMD) are leading causes of blindness. Current treatments only slow disease progression in a minority of patients, therefore it is important to develop new treatments that can regenerate lost cells, restore retinal function and halt disease progression. Retinal progenitor cells have the potential to replace degenerating photoreceptors in retinal diseases. This thesis explores the viability of using immortalised human foetal retinal progenitor cells for this purpose, and aims to elucidate the trophic factors required, *in vitro*, to drive these cells towards retinal cell lineages. We also examine their potential to survive, integrate and differentiate in the diseased and developing rat retina. Two human foetal retinal progenitor cell lines were established by infecting retinal foetal tissue with the temperature-sensitive tsT-SV40 antigen. The immortalised progenitor cells were compared to primary human foetal retinal progenitor cultures, and both were shown to express similar markers of neural progenitor cells and early neuronal cell types. Several trophic factors were investigated, including serum, retinoic acid and conditioned medium, with respect to their ability to influence gene expression. Serum appeared to induce expression of ganglion cell markers, and conditioned medium stimulated cell proliferation. Cells were also grafted into neonatal hooded Lister rats and RCS dystrophic rats, and neither the primary nor the immortalised progenitor cells demonstrated integration into the retina. In the RCS rat, cells were engulfed by host macrophage/microglia and showed no signs of integration or expression of neuronal markers. Immortalised cells, *in vivo*, stained positively for Ki67 and low levels of nestin, but exhibited limited ability to differentiate or integrate. These data indicate that immortalised progenitor cells maintain many characterisics of unimmortalised progenitor cells, and suggest that immortalisation of retinal progenitors may have long term therapeutic possibilities.

Declaration

I confirm that the work presented in this thesis is my own. Where information has been derived from other sources I confirm that this has been indicated in the thesis.

Shazeen M. Hasan

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AAV	adeno-associated virus
AMD	age-related macular degeneration
°C	degrees celsius
bFGF	basic fibroblast growth factor
BMP	bone morphogenetic protein
bp	base pair
cDNA	complementary DNA
CMZ	ciliary marginal zone
CNS	central nervous system
Crx	cone rod homebox
CO_2	carbon dioxide
DAPI	4', 6-diamidino-2-phenylindole dihydrochoride
DEPC	diethyl pyrocarbonate
ddH_2O	double distilled water
DMEM	dulbecco's modified eagle medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxyribonucleotide (5'-) triphosphates
EDTA	ethylene diamine tetra acetic acid
EFTFs	eye field transcription factors
EGF	epidermal growth factor
ERG	electroretinography
FBS	foetal bovine serum
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
FSC	forward scatter light
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GCL	ganglion cell layer
h	hour
HCl	hydrochloric acid
HEPES	hydroxyethylpiperazine-N'-2-ethanesulfonic acid

INL	inner nuclear layer
IPL	inner plexiform layer
Kb	kilobases
M	molar
mol	moles
mg	milligram (10^{-3} g)
µg	microgram (10^{-6} g)
µl	microlitre (10^{-6} L)
µm	micrometer (10^{-6} m)
ml	millilitre (10^{-3} L)
µM	micromolar (10^{-6} M)
mM	millimolar (10^{-3} M)
$MgCl_2$	magnesium chloride
Mitf	microphthalmia associated transcription factor
mRNA	messenger RNA
NaCl	sodium chloride
NaOH	sodium hydroxide
NDS	normal donkey serum
NF160	neurofilament 160kD
NF200	neurofilament 200kD
ng	nanogram (10^{-9} g)
NHS	normal horse serum
nmol	nanomole (10^{-9} M)
NRL	neural leucine zipper
ONL	outer nuclear layer
OPL	outer plexiform layer
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PD	parkinson's disease
PE	phycoerythrin
PFA	paraformaldehyde
pg	picogram (10^{-12} g)
pmol	picomole (10^{-12} M)
PMSF	phenyl-methylsulphonyl-fluoride
Ptc	patched

RA	retinoic acid
RAR	retinoid A receptor
RARE	retinoic acid responsive DNA elements
Rb	retinoblastoma gene
RNA	ribonucleic acid
RP	retinitis pigmentosa
RPE	retinal pigment epithelium
rpm	rotations per minute
RT	reverse transcriptase
RT-PCR	reverse transcriptase-PCR
RXR	retinoid X receptor
RT-PCR	reverse transcriptase-PCR
s	seconds
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
Shh	sonic hedgehog
SSC	side scatter light
SV40	simian virus 40
TAE	tris-acetate-EDTA
TBS	tris-buffered saline
TE	tris-EDTA
TEMED	tetra-methylethylene-diamine
TRITC	tetramethyl-rhodamine isothiocyanate
UV	ultraviolet
V	volts
v/v	volume per volume
Wnt	wingless-Int
w/v	weight per volume

Chapter 1

Introduction

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Introduction

1.1 General introduction

The inherited retinal dystrophies form a large group of diseases that are collectively known as retinitis pigmentosa (RP). The recent discovery of mutations in complement factor H and factor B that contribute to the development of age-related macular degeneration (AMD), means that the genetic basis of blinding eye diseases is beginning to be elucidated. Almost 50% of blindness in Europe is attributed to AMD alone (Resnikoff *et al.*, 2004), and within the UK approximately 1% of the population is affected by the condition (figure from The Macular Disease Society). The primary defect lies in the neural retina, where the photoreceptors and retinal pigment epithelium (RPE) are mainly affected. Treatments that are currently available only manage to slow disease progression in a minority of patients. Therefore, it is increasingly important to explore and develop new treatments for retinal degenerative diseases that can regenerate lost cells to restore retinal function and halt disease progression. An increasingly exciting avenue of research is the use of retinal stem and progenitor cells as a replacement for degenerating or dysfunctional cells. This could eventually lead to the amelioration of many retinal dystrophies in a significant proportion of the population. A major advantage in studying the retina is the accessibility of the intact tissue and the fairly autonomous development it exhibits compared to other areas of the central nervous system (CNS) (Cepko *et al.*, 1996). Studies investigating the use of cellular replacement to recover vision in animal models have shown promise (Gouras *et al.*, 1989; Little *et al.*, 1996; Coffey *et al.*, 2002), however several issues need to be addressed such as the cell type used for transplantation and how to enhance the survival and integration of such cells.

This thesis explores the viability of using immortalised human foetal retinal progenitor cells in a degenerative environment, as a possible cellular replacement therapy to alleviate the progression of disease. Assessments will be carried out *in vitro* to examine the capacity of such cells to differentiate into retinal cell types, with a particular focus on the rod photoreceptor lineage. By trying to understand the mechanisms underlying photoreceptor development this knowledge can then be manipulated to 'direct' the differentiation of

various retinal progenitor cells, in the hope that this strategy may eventually be used to repair tissue structures and replace lost function.

1.2 Anatomy of the eye

The eye is an intricate organ (figure 1.1) that converts light energy from our surroundings into nerve action potentials, in specialized nerve cells (rods and cones) in the retina. The action potentials eventually travel to the brain where they are converted and consciously appreciated as vision (Forrester *et al.*, 2002). This process is known as phototransduction, and all structures in the eye are necessary to support it. The eye is composed of three tunics (layers), these being the corneoscleral layer, the vascular or uveal tract and the neural layer (the retina). The corneoscleral layer is a fibrous coat composed of the posteriorly positioned opaque sclera, and the transparent cornea covering the anterior surface of the eye. This 'coat' gives protection and structural support to the eyeball. The vascular tunic is formed from the choroid, ciliary body and iris. The iris functions to regulate pupil size to obtain optimum visual capacity. The retina is the innermost of the three tunics and lines the back of the eye. The neural retina, itself, is subdivided into three laminar structures: the ganglion cell layer (GCL), the inner nuclear layer (INL) and the outer nuclear layer (ONL).

1.3 Development of the vertebrate eye primordia

The intricate circuitry and structures that form the eye rely on close contact between neighbouring cells to enable communication of inductive signals and to enable these and other cells to have the functional plasticity to respond to diverse inputs and stimuli (Gilbert, 2000). The organogenesis of the eye begins in the fourth week post fertilisation. Prior to this stage the fertilized zygote undergoes cleavage which involves the successive division of cells without any increase in size. This eventually leads to gastrulation, involving the movement of cells resulting in the three germ layers, (ectoderm, mesoderm and endoderm) situated in the correct locations for ongoing development (Wolpert *et al.*, 1998).

The initial stage of CNS formation begins with the thickening of the dorsal region of the surface ectoderm, which results in the formation of neural plates. By day 18, at the mid-region of the neural plates, neural grooves migrate inwards, causing the bordering neural folds to converge towards one another to forms a hollow tube. Neurulation follows,

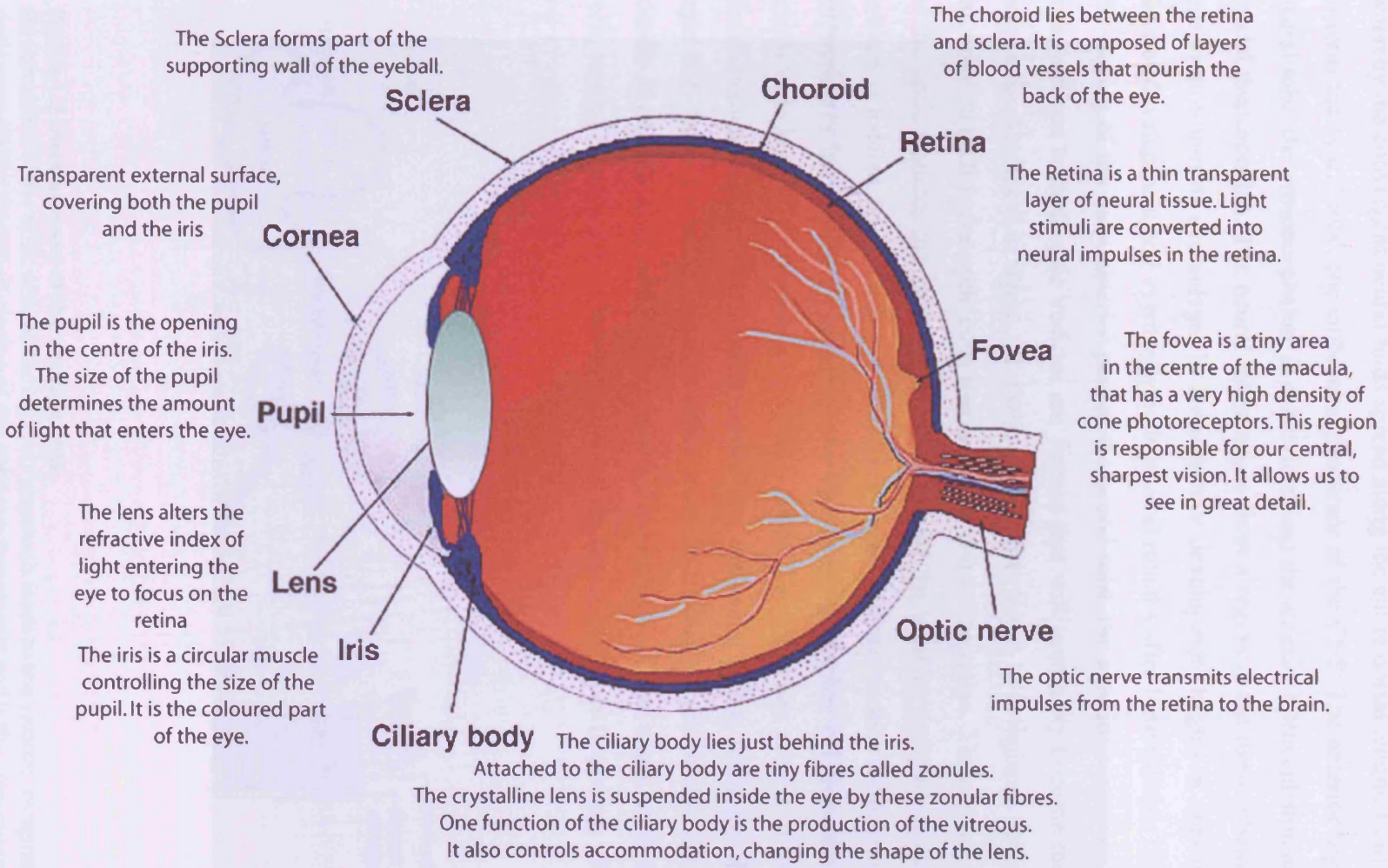

Figure 1.1 Sagittal section of the human adult eye
Captions identifying the main components of the human adult eye and their respective functions.
(Adapted from Webvision; http://www.webvision.med.utah.edu/)

whereby the convergent neural folds spread along the entire dorsal length, from head to toe, to form the neural tube, the embryonic precursor of the CNS. The anterior segment of the neural tube, the prosencephalon, is subdivided into the anterior telencephalon and the more caudal diencephalon. The neural retina arises from a region in the diencephalon called the eye primordium or eye anlage. In humans, eye development begins at approximately 22 days of development and eventually ends several months after birth (Mann, 1964). Prior to the closing of the most anterior part of the neural tube, the anterior neuropore, two lateral invaginations termed optic vesicles are formed that will eventually become the future eye primordia. As shown in figure 1.2, these vesicles then go on to invaginate further and form the optic cups. It is the optic cups that will give rise to the retina. Throughout the forming of the optic vesicles the lateral convex regions of the vesicles maintain contact with the surface ectoderm (Bron *et al.*, 1997). This region of the surface ectoderm eventually differentiates into the lens placode of the eyes when it pinches off from the rest of the ectoderm to become the lens vesicle (Wong, 2006). The thickened portion of the optic cup, that invaginates, is destined to differentiate into the neural retina, while the thinner outer layer will form the retinal pigment epithelium (RPE). At the site of the cup, successive rounds of cell division occur to generate a neuroepithelial layer many cells thick. These cells, termed neuroblasts, differentiate into all the retinal cell types (Dowling, 1987).

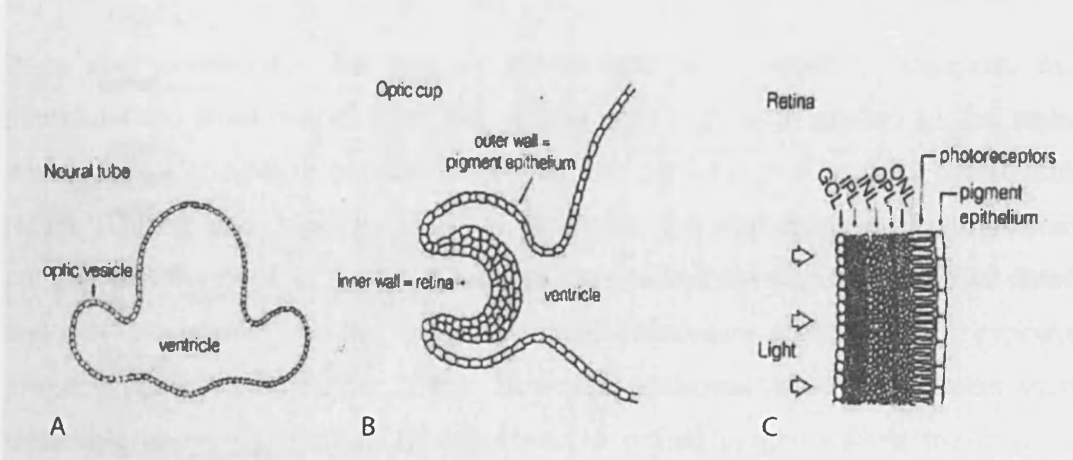

Figure 1.2 Development of the neural retina
(A) Formation of the optic vesicles during embryogenesis leads to the vesicles invaginating to form optic cups. (B) The thickened portion of the optic cup invaginates and is the presumptive neural retina whilst the outer wall will differentiate into the retinal pigment epithelium. (C) A schematic of the developed neural retina. (Adapted from Dowling, 1987).

1.3.1 Retinal specification

During eye development, inductive cues exist to specify the lineage that individual cells adopt, and as development progresses, the lineage options become increasingly restricted as partial and then full commitment determine cell fate. The interesting question is when such restrictions come into play for cells destined to become part of the pool of definitive retina-producing progenitor cells. Exploratory studies using Xenopus and zebrafish embryos, have tried to identify the location of eye-forming cells within the embryo. With the use of fluorescent dye injections, it was found that prior to gastrulation, tissue contribution from a particular blastomere was largely undetermined (Kimmel and Warga, 1987). Following the work of Spemann and Mangold it was found that transplantation of the presumptive neural ectoderm from one newt embryo to the presumptive epidermis of another embryo, gave rise to ectopic neural plates when the transplant was taken from an embryo in the late-gastrula stage but not before (Gilbert, 2000). However, quantitative lineage analysis in Xenopus embryos, determined that each retina descends from a stereotypic subset of 9 animal pole blastomeres at the 32 cell stage (prior to gastrulation), that acquire the competence to contribute to the retina (figure 1.3). These blastomeres have been shown to be biased towards retinal lineage development, however they are not committed as yet (Zaghloul *et al.*, 2005).

It is also noteworthy that not all blastomeres were equally competent, even after manipulation. Blastomeres from the vegetal pole that were grafted to the animal pole, where retinal competent blastomeres reside, did not have any progeny contributing to the retina (Huang and Moody, 1993). Even with the introduction of various signalling components involved in neural induction, they lacked the intrinsic maternal determinants and cell-cell interactions that bias the animal blastomere counterparts to produce retinal progeny (Zuber and Harris, 2006). However equatorial blastomeres seem to be more malleable to reprogramming to contribute to retinal progeny when transplanted to the animal pole (Huang and Moody, 1993). These experiments have identified a period prior to gastrulation where retinal-competence in blastomeres is exhibited, but this does not specify the eye field at this stage. Experiments involving the transplantation of different regions of the presumptive neural plate, in mid-gastrula Xenopus embryos, have shown that all regions of the neural plate are capable of producing eyes. These observations indicate that

specification of the eye region is not firmly fixed until late gastrulation (Saha and Grainger, 1992).

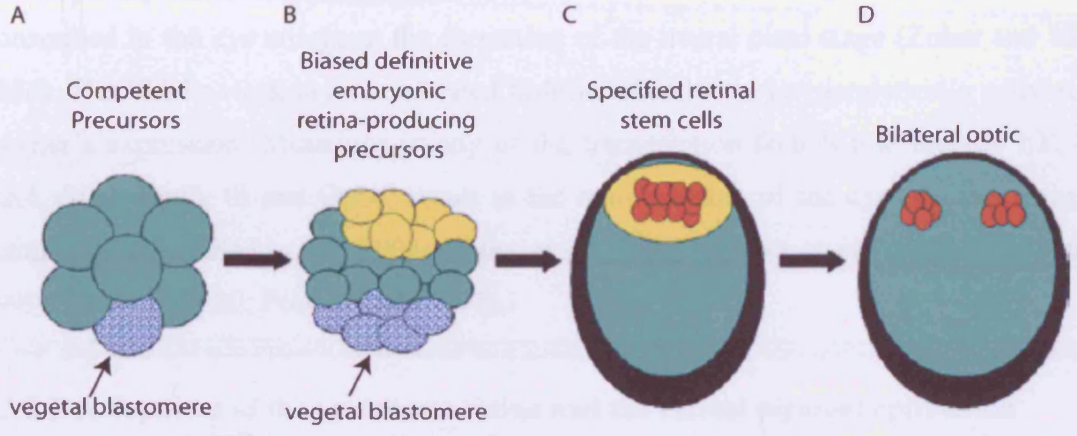

Figure 1.3 Specification of the eye primordia and retinal stem cells in the Xenopus embryo
Retinal development consists of several steps. (A) First a subset of embryonic blastomeres (green) are competent to contribute to the retina, whilst the vegetal blastomeres (blue hatched) are inhibited from forming retina. (B) A smaller subset of the competent blastomeres (yellow) are biased to become the definitive retina-producing precursor cells in response to retinogenic signals. (C) Retinal stem cells (red) become specified from the descendants of the definitive retina-producing cells (yellow) and form the eye field at the anterior end of the neural plate (green). (D) During neurulation, the retinal stem cells separate into the right and left optic primordia. During neural tube morphogenesis each optic primordium will eventually give rise to an optic vesicle which in turn will give rise to the optic stalk, retinal pigment epithelium and neural retina, each containing region-specific retinal progenitor cells. (Adapted from Zaghloul *et al.*, 2005).

1.3.2 Signalling pathways determining formation of the eye primordia

Retinal tissue is competent prior to gastrulation but is defined during gastrulation, a process that involves a plethora of signalling pathways to orchestrate ocular development. Due to the conserved nature and gene expression patterns of the retina from Drosophila to humans, a great deal of knowledge has been extracted from studies in lower vertebrates. Wingless-Int (Wnt) proteins play a pivotal role during ocular development alongside the antagonism of bone morphogenetic protein (BMP). BMP antagonism is necessary for the retinogenic animal blastomeres to go on to generate retinal lineages. When ectopic expression of BMP

occurs in these blastomeres they fail to produce retinal progeny (Zaghloul *et al.*, 2005). This concurs with the need for BMP inhibition in neural induction. The antagonist of BMP signalling is the neural inducer noggin. Studies in Xenopus report that over-expression of Wnt8b results in complete loss of eye development, thus confirming that inhibition of Wnt is required for correct neural plate development (Kim *et al.*, 2007). A conserved set of eye field transcription factors (EFTFs) are also essential for normal eye development and are transcribed in the eye anlage at the formation of the neural plate stage (Zuber and Harris, 2006). The EFTFs work in a coordinated fashion whereby they synergistically activate one another's expression. Mutations in any of the transcription factors that include ET, Rx1, Pax6, Six3, Lhx2, tll and Optx2 result in the malformation of the eye or loss of the eye completely (Voronina *et al.*, 2004; Chow *et al.*, 1999 Lagutin *et al.*, 2003; Zuber *et al.*, 1999; Yu *et al.*, 2000; Porter *et al.*, 1997).

1.3.3 Development of the vertebrate retina and the retinal pigment epithelium

Once the eye primordia have been identified and the invagination of the optic vesicle has formed the optic cup, these neuroblasts must make a key choice as to their fate which is to be either the neural retina or the retinal pigment epithelium (RPE). The surface ectoderm (presumptive lens) plays a role in neural retinal specification, as the removal of the surface ectoderm results in inhibition of neural retinal development (Hyer *et al.*, 1998). There exist three lines of evidence to suggest that members of the fibroblast growth factor (FGF) family contribute to the development of the neural retina. Firstly, the addition of neutralizing FGF2 antibodies to chick optic vesicles inhibits neural development (Pittack *et al.*, 1997). Secondly, when the surface ectoderm is removed from developing optic vesicle, the addition of FGF-coated beads is sufficient for neural development to continue (Hyer *et al.*, 1998), implying that one of the factors secreted by the surface ectoderm could be FGF. Finally, the presumptive RPE is capable of transdifferentiating into neural retina when cultured in the presence of FGF (Park and Hollenberg, 1989). As a result of the inductive signals from the surface ectoderm, the expression of one of the first transcription factors, Chx10, a member of the paired-like homeobox gene family, is up-regulated in the presumptive neural retina. This transcription factor is essential in eye development as mutations result in microphthalmia. The role of Chx10 seems to be in the regulation of cell proliferation as opposed to the differentiation of the neural retina (Burmeister *et al.*, 1996).

However, Chx10 is required for the down-regulation of another transcription factor, namely microphthalmia associated transcription factor (Mitf) which is involved in the maintenance of RPE cell identity (Vugler *et al.*, 2007). Mitf expression is localized to the presumptive RPE and the dorsal aspect of the optic cup, but Chx10 expression down-regulates Mitf in the presumptive neural retina (Chow and Lang, 2001). Conversely, Chx10 is suppressed by signals emitted from the mesenchyme surrounding the eye that enhance Mitf expression (Fuhrmann *et al.*, 2000). Remarkably, just as the RPE can transdifferentiate into neural retina the opposite occurs in Mitf loss-of-function mutants, suggesting that Mitf down-regulation is necessary for the specification of the neural retina (Bumsted and Barnstable, 2000). In the developing retina a dynamic interplay exists between Chx10 and Mitf expression that results in an interdependence of specification between the neural retina and the RPE.

1.3.4 Development and organisation of the vertebrate retina

The work begun many years ago by Santiago Ramon y Cajal has led to the identification of six major classes of neuronal cells (ganglion, amacrine, bipolar, horizontal, cone- and rod-photoreceptors) and one type of glia (Müller glia) that comprise the neural retina. The neural retina is composed of three layers of cell bodies: the outer nuclear layer (ONL) which contains the cell bodies of the photoreceptors (rods and cones), the inner nuclear layer (INL) which contains the bipolar, horizontal and amacrine cells, and the third layer, comprising ganglion cells (GCL) and displaced amacrine cells. Two layers of synapses, found at the outer (OPL) and inner plexiform layers (IPL) connect the three layers of the neural retina. Within the OPL, photoreceptors synapse with bipolar cells, which in turn synapse with ganglion cells within the IPL. Bipolar cells also synapse with horizontal and amacrine cells forming further networks that influence and integrate the ganglion cell signals. The visual signal is transmitted from the back of the eye towards the ganglion cell axons that carry the signal to the visual cortex of the brain, where it is further processed and interpreted (Kolb, 2003). Neurons in the developing retina arise from multipotent progenitor cells largely in response to local cell-cell interactions in an evolutionarily conserved temporal and spatial pattern (figure 1.4) (Ahmad *et al.*, 2000). Ganglion cells, cone photoreceptors, amacrine cells and horizontal cells are born in the first wave, and bipolar neurons, rod photoreceptors and Müller glia are generated in the second wave of differentiating cell types (Marquardt and Gruss, 2002).

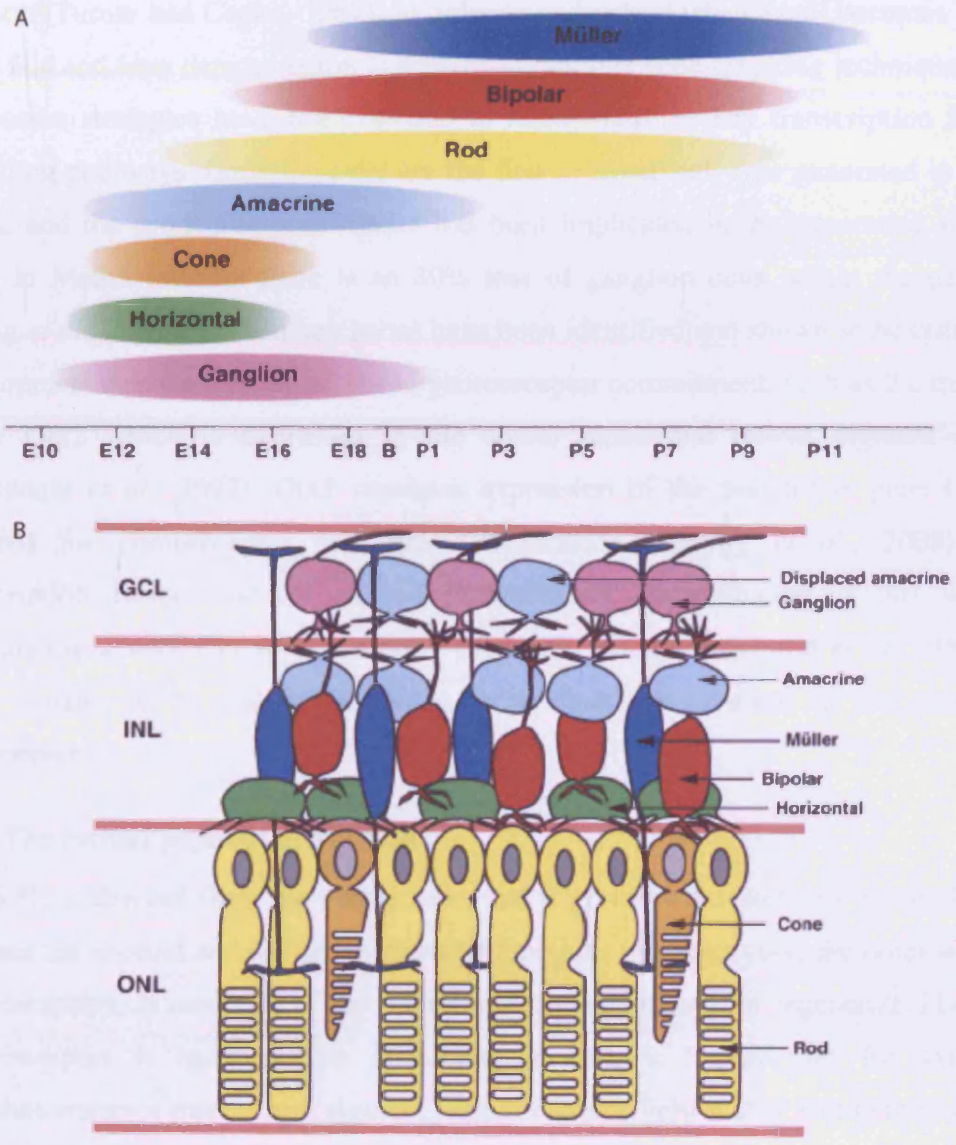

Figure 1.4 Stereotypic formation and structure of the neural retina
(A) Temporal generation of the mammalian retinal cell types showing the stereotypic order of retinal cell development with the ganglion cells generated first and the Müller glial cells generated last. (B) Schematic representation of the laminar structure of the neural retina. The neural retina consists of three laminar layers- GCL: ganglion cell layer; INL: inner nuclear layer and ONL: outer nuclear layer.
(Adapted from Ohsawa and Kageyama, 2007).

1.3.5 Signalling pathways required for neural retinal development

Many secreted factors and signalling pathways are involved in the development of the neural retina. Lineage analysis in the rat has revealed that progenitor cells within the retina are multipotent and can generate several different retinal cell types up until their final cell division (Turner and Cepko, 1987). In order to understand when a cell becomes committed to its fate and how determination is controlled, various gene-targeting techniques and mis-expression strategies have been devised to manipulate the key transcription factors and signalling pathways. Ganglion cells are the first neuronal cell type generated in the neural retina, and the proneural gene Math5 has been implicated in the generation of ganglion cells. In Math5 mutants there is an 80% loss of ganglion cells within the neural retina (Wang *et al.*, 2001). Certain key genes have been identified and shown to be critical for the developmental process involved in rod photoreceptor commitment, such as the transcription factor Otx2 which is expressed in the neural retina and retinal pigment epithelium (Bovolenta *et al.*, 1997). Otx2 regulates expression of the homeobox gene Crx that is required for photoreceptor cell fate determination (Hennig *et al.*, 2008). Another transcription factor essential in rod photoreceptor determination is Nrl which acts synergistically with Crx to activate the rhodopsin promoter (Mitton *et al.*, 2000). Much more remains to be understood about the mechanisms involved in rod photoreceptor development.

1.3.6 The retinal pigment epithelium

The RPE is derived from the neural tube, and it provides a selectively permeable barrier between the choroid and the neural retina. It functions to phagocytose the outer segments of photoreceptors, is essential in the phototransduction pathway to regenerate 11-cis retinal and transport it back to the photoreceptor cell, it is the site for synthesis of interphotoreceptor matrix, and pigment granules reduce light scatter within the eye (Rattner *et al.*,1999; Forrester *et al.*, 2002).

1.3.7 Photoreceptor cells

The human eye contains two types of photoreceptors, rods and cones, which make up the outer layer of the neural retina. Rods, which account for approximately 95% of photoreceptors in humans, are the cells involved in low light vision, whereas the 5% of cone cells are responsible for us visualising objects in bright light. The density of the rod

and cone cells varies within the retina. The peripheral regions are dominated by rod cells whereas in the centre there is a greater concentration of cones, especially at the region exclusive for cone cells in the centre of the macula- the fovea. Each photoreceptor is composed of a long narrow cell with an inner and outer segment joined together by a connecting stalk or cilium. Contained within the outer segments are the visual pigments, rhodopsin in rod cells, and green, red and blue opsins in cones. It is these pigments that are responsible for the absorption of light, which leads to the generation of electrical impulses to the brain, a series of events collectively known as phototransduction.

1.3.8 Phototransduction

Phototransduction occurs in the outer segment of the retinal photoreceptor cells. The reaction cascade amplifies cellular responses to external signals, photons of light, received from the environment. The receptor rhodopsin is a heptahelical transmembrane protein, which has a covalently linked 11-cis-retinal chromophore. It is the chromophore that becomes activated by light and which then enables rhodopsin to activate the G-protein, transducin. Transducin is a multi-subunit protein composed of three chains, α, β and γ. G-proteins are in the OFF state when the α subunit binds to a GDP molecule, and are switched ON when GTP occupies the binding site. Therefore, rhodopsin is the receptor necessary to switch on transducin and cause the attachment of GTP to the α subunit. When bound, transducin has the ability to hydrolyse GTP, and this reaction consequently activates the effector enzyme cGMP phosphodiesterase (PDE). In its inactive state PDE is a heterotetramer consisting of α and β subunits bound to a γ subunit. Upon binding of the GTP-bound α-transducin subunit, PDE undergoes a conformational change leading to the removal of the inhibitory γ subunit. The two remaining subunits hydrolyse cGMP bound to the rod photoreceptor cGMP-gated channel, to 5'GMP, a side product of this reduction reaction being protons. This reaction induces the closure of Na^+ and Ca^{2+} channels in the plasma membrane of the outer segment. The resulting hyperpolarisation of the cellular membrane generates the photoreceptor signal. A reduction in the intracellular level of calcium stimulates recoverin, which in turn activates guanylate cyclase. Guanylate cyclase synthesizes cGMP from GTP thereby restoring the outer segment cGMP level to baseline, and consequently the reopening of the cGMP-gated channel. In addition, phosphorylation of rhodopsin by rhodopsin kinase (a photoreceptor specific enzyme) leads to its

inactivation. Both events return rod cells to their ground state. Phototransduction in cones utilizes proteins that are homologous but distinct to those found in rods.

1.4 Retinal dystrophies

With such an intricate pathway responsible for vision, it is not surprising that a problem within the eye can lead to devastating effects, for which few therapeutic remedies currently exist. There are numerous disorders which cause the degeneration of photoreceptors and RPE, two that have a major impact on the world's population are discussed here.

1.4.1 Retinitis pigmentosa

Retinitis Pigmentosa (RP) constitutes a group of heterogeneous inherited dystrophies that have an estimated prevalence of between 1 in 3000 and 1 in 5000 worldwide (Haim, 2002). The predominant characteristics that all these dystrophies share are a loss in visual acuity from the periphery, that gradually progresses towards the macula, and a bilateral involvement. Several genes have been implicated in the onset of RP, resulting in a range of genetic mutations that cause photoreceptor apoptosis, often leading to functional blindness (Kalloniatis and Fletcher, 2004). The predominant gene mutations afflict the rhodopsin gene, however there are mutations in genes that are involved in the phototransduction cascade such as transducin and rhodopsin kinase. The modes of inheritance in RP range from autosomal dominant, x-linked, and simplex to multiplex. The simplex and multiplex groups are likely to be due to autosomal recessive inheritance (Tyler *et al.*, 1984). However, it is believed that environmental interactions could also play a role in simplex forms of RP (Stone *et al.*, 1999). Most patients present with night blindness as the initial sign of RP in their early teens, which leads to progressive reduction in peripheral vision. The eventual loss of central vision is often observed in the fifth to seventh decades, but in severe RP the loss of central vision may be much earlier. Potential therapies for RP include the administration of various growth factors including basic fibroblast growth factor (bFGF), which has shown to slow photoreceptor loss in a range of rodent models including the RCS rat (Faktorovich *et al.*, 1990; Perry *et al.*, 1995). Modifications in diet may be useful in some retinal dystrophies.

1.4.2 Age-related macular degeneration

Age-related macular degeneration (AMD) is a leading cause of blindness in the developed world that increases significantly with age (Tuo *et al.*, 2004). AMD affects the macula, the region of the eye containing cone photoreceptors that is responsible for visualising detail, and when these cells degenerate only peripheral vision remains (Feret *et al.*, 2007). In AMD the primary cause is clearly linked to RPE dysfunction (Beatty *et al.*, 2000), leading to gradual degeneration of Bruch's membrane, photoreceptors and the choriocapillaris in the macula (Fine *et al.*, 2000). There are two grades of AMD, one being wet AMD, in which abnormal blood vessels start to grow behind the retina underneath the macula, causing rapid damage to the retina, and the second being dry AMD, when the cone photoreceptors in the macula break down, leading to cone atrophy. At least three genes have been implicated in AMD; complement factor H (CFH), complement factor B and age-related maculopathy susceptibility 2 (ARMS2) (Patel *et al.*, 2007; Kanda *et al.*, 2007). Risk factors other than age include positive family history, diet and environmental factors, especially smoking (Hyman and Neborsky, 2002). AMD will become more prevalent as the life-span of humans continues to increase, and as 30% of 75 year olds and older have a form of AMD the incidence of AMD is expected to double within 25 years (Smith *et al.*, 2001). The therapies available to date to limit disease progression include surgical macular translocation, and anti-angiogenesis agents to prevent blood vessel growth and leakage in wet AMD. With respect to both RP and AMD, ganglion cell output neurons are largely unaffected (Medeiros and Curcio, 2001; Stone *et al.*, 1992). This is important if cellular replacement is to become a reality, otherwise functional vision could not be restored.

1.5 Possible therapies

Alongside the administration of drugs and translocation surgery that are currently being used to treat the increasing numbers of patients with retinal degenerations, there are newer therapies in clinical trials that could one day hold greater promise in treating these disorders.

1.5.1 Gene therapy

Gene replacement therapy is theoretically capable of creating missing cellular components when genetic mutations interfere with the development or function of a photoreceptor cell. The *rds* rodent model has mutations in the *Prph2* gene resulting in a severe photoreceptor dystrophy, and in humans *Prph2* mutations lead to the autosomal recessive condition, Macular Dystrophy. Using electroretinography (ERG), a diagnostic tool that measures photoreceptor cell function, 10 week old *rds* mice treated with subretinal injections of recombinant adeno-associated virus (AAV) encoding a *Prph2* transgene, had significant ERG recordings, indicating improvements in retinal function (Ali *et al.,* 2000). These results were compared with untreated *rds* mice of the same age, which showed undetectable ERG responses. Gene therapy will not be suitable for all retinal degenerative diseases, and is only likely to be applicable to autosomal recessive diseases and some x-linked diseases. With regards to RP, 31 genes have been implicated, however each known mutation only accounts for less than 10% of RP patients (Wang *et al.*, 2005). However in AMD, known mutations can now explain more than 50% of disease. Due to the multitude of mutations responsible for visual deficits, the application of gene therapy in the long-term may be severely constrained.

1.5.2 RNA interference

For dominant autosomal diseases, small interfering RNA (siRNA) may be of therapeutic benefit. In dominant forms of a disorder the heterozygous patient carries a mutant allele of a gene and a healthy functioning allele of the same gene. Mutant alleles produce dysfunctional, sometimes toxic proteins that damage photoreceptors. siRNAs have the ability to disrupt the mutant allele's ability to produce harmful proteins, by precipitating cleavage of the target mRNAs (Farrar *et al.,* 2002). siRNAs are 21-23 nucleotide long double-stranded RNA (dsRNA) molecules with 2 nucleotide overhang at the 3' terminal end of the structure (Pushparaj and Melendez, 2006). The pathway involved in RNA interference (RNAi) mediated by siRNAs involves the introduction of dsRNA into the target cells, where the dsRNAs are then converted into siRNA by an RNase III-like enzyme called Dicer. Subsequently, the siRNAs unwind and enter the RNA-induced silencing complex (RISC) that allows the cellular RNA that is complementary to the single stranded siRNA, to be targeted to undergo enzymatic degradation or inhibition of translation. This

results in inhibition of translation of the gene product from the diseased allele, but it allows transcripts from the healthy allele to be processed and translated normally and thus produces the required protein. Notwithstanding the problems of haploinsufficiency that are known to exist for rhodopsin, this approach may nevertheless limit disease progression.

This form of therapy has already shown some benefit in the effective targeting and inhibition of vascular endothelial growth factor (VEGF) in a mouse model. The results showed that blocking endogenous production of VEGF protected the animals from choroidal neovascularization, this being a major problem in the wet form of AMD (Reich *et al.*, 2003). However, a limitation to this therapeutic strategy is the method in which the delivery of siRNAs into the target cell/tissue is administered. To date the most efficacious method has been to use viral vectors because of their high transfection efficiency and ability to carry siRNAs into non-dividing cells such as neurons (Pushparaj and Melendez, 2006). However the use of viral vectors in humans has raised safety concerns. Therefore until a safe and reliable delivery method is obtained there is a constraint on RNA interference as a therapeutic intervention.

Something neither gene nor siRNA-based therapies can accomplish is the regeneration of photoreceptors once the cells have died, however one attractive possibility is to use specific cells or tissue to replace lost photoreceptor cells and accessory cells, to reverse the loss of function and halt disease progression.

1.5.3 Cellular therapy

The use of neural transplantation as a means of treating disease of the CNS was first attempted by Thompson (1890), when neuronal tissue was grafted into an adult dog brain. More than a century later, this approach has led to some success in treating Parkinson's disease (PD), by grafting dopaminergic cells into the brains of patients (Gray *et al.,* 1999). Ideally, cellular therapy would not only replenish damaged cell types but be a means of introducing growth factors to induce a more receptive environment for regeneration. By transplanting cells, one would hope this would also activate endogenous growth factors and cytokines, and in turn would have a knock-on effect by stimulating the host's own neural stem or progenitor cells to aid the repair of damage and deficit. If cellular therapy could be harnessed to fulfil all these requirements, it would provide a therapeutic option even more

efficacious than originally believed. Unfortunately, the need for transplantable cells far outweighs the supply, as the use of adult tissue grafts is neither consistent nor renewable. The logical step is to look elsewhere, and there is now an intense interest in embryonic and foetal tissues, and in particular stem and progenitor cells that originate from them. However, no solution comes without its share of ethical and technical issues. As organisms develop, stem cells become an increasingly sparse population, which makes them more difficult to locate, as well as to study and understand (Temple, 2001). The ideal cell type for cellular therapy should be obtained easily and consistently cultured *in vitro*, until the time came to differentiate the cells into the desired cell type. Once grafted into the host, the cells should not elicit a host immunological response or develop into a tumour. Most importantly, cells should have the ability to integrate, survive and function in the host tissue or organ. This has led the field of transplantation, particularly neural transplantation, to advance our understanding of neural stem cells. All the therapeutic methods outlined here have potential, though unfortunately, none are yet close to being problem free.

1.5.4 Retinal remodelling and cellular therapy

A degenerating retina typically shows signs of modification within the neural-retinal structure, leading to the development of neurite remodelling and the establishment of rogue circuitry (Marc *et al.,* 2003a; Marc *et al.*, 2003b). Inevitably, this can lead to problems with retinal transplants, therefore the time at which transplantation occurs is paramount. However, the ability of the retina to remodel shows the plasticity inherent within the neural retina. If these mechanisms were better understood they might be harnessed to benefit retinal transplantation studies.

1.6 Stem cells and progenitor cells

There remains a good deal of ambiguity surrounding the exact definition of a stem cell. In general, a stem cell has both the capacity to self-renew and to generate differentiated progeny by symmetric and asymmetric proliferation (Morrison *et al.*, 1997). Human embryonic stem cells are pluripotent by nature, meaning they can give rise to all cells that arise from the three germ layers, (endoderm, mesoderm and ectoderm), but not the embryo because they are unable to give rise to the placenta and supporting tissues. Stem cells are concentrated in specific regions of the developing vertebrate. Consequently, they become

established in adult tissues and are continually regenerating host tissue, such as blood, skin, nasal epithelium and intestine (Temple, 2001).

Although stem cells are functional in regions of the adult central nervous system, their presence until recently has been largely disregarded. How and why would an area apparently incapable of regeneration house stem cells and their regenerative capacity? A possible reason for this dichotomy is that stem cells exist in microenvironments or niches that maintain the cells in a quiescent state, safeguarding them from factors that could induce the cells to differentiate (Morrison *et al*, 1997). Stem cells in the CNS are capable of generating neurons, astrocytes and oligodendrocytes, and they can self-renew and undergo asymmetric division to give rise to cells other than themselves (McKay, 1997; Gage, 2000). However, in this thesis the focus is a class of retinal progenitor cells that possess a more restricted potential than stem cells. Retinal progenitor cells are multipotent in that they are capable of generating the neurons and glia that make up the neural retina (Ahmad *et al.*, 1999). During development, after a given number of cell divisions progenitors usually exit the cell cycle and differentiate into neurons and glia, however some continue to persist in a progenitor state. There has been a mass of activity surrounding the location and identity of the progenitor cells capable of neurogenesis in the embryonic and postnatal mammal, following Altman's first observations of neurogenesis in the adult brain (1969).

1.6.1 Discovery of stem cells in the ciliary marginal zone

In 1781, Claude Bonnet discovered that adult newts possess the ability to regenerate their eyes following the removal of part of the eye (Leigh-Close and Reh, 2006). This led to the discovery that fish and amphibians have the enviable ability to regenerate retina throughout their lifetimes, and thus began the investigation into the cell types that mediate this regeneration. An intracellular tracer of fluorescent dextran was used to demonstrate that individually microinjected cells within the eyes of larval Xenopus gave rise to descendants that differentiated into all the major cell types required to compose the retina (Wetts, *et al.*, 1989). The cells that contributed to the growth of the retina postnatally were found at the peripheral margin of the eye known as the ciliary marginal zone (CMZ) or *ora serrata* (figure 1.5). These cells allow the growth of the retina via the addition of concentric circles of cells, where the youngest and least developed cells are closest to the CMZ. These cells are the self-renewing neural stem cells which then give rise to more multipotent progenitor

cells directly next to them. This eventually leads to retinoblasts that are restricted in cell fate, and finally the furthest cells from the CMZ but still within the region of retinogenesis, that have stopped proliferating and started to differentiate (Perron and Harris, 2000). Exploratory studies in the avian eye resulted in the detection of mitotically active cells, showing that new neurons are generated for at least 2 months after hatching. When these cells were further analysed they were found to express markers that are found in retinal progenitors including Chx10 and Pax6. This work showed that the chick has ongoing neurogenesis at the CMZ similar to that in lower vertebrates. However, one difference that exists between the CMZ of post-hatch chicks and lower vertebrates is the inability of the chick CMZ to produce all the retinal neurons, unlike the lower vertebrates that can generate all the retinal neurons required to regenerate a retina. This discrepancy is not due to an intrinsic limitation, but exists because the microenvironment lacks the relevant cues. Once certain factors were added, other neuronal cell types were induced (Fischer and Reh, 2000; Kubota *et al.*, 2002; Fischer and Reh, 2003).

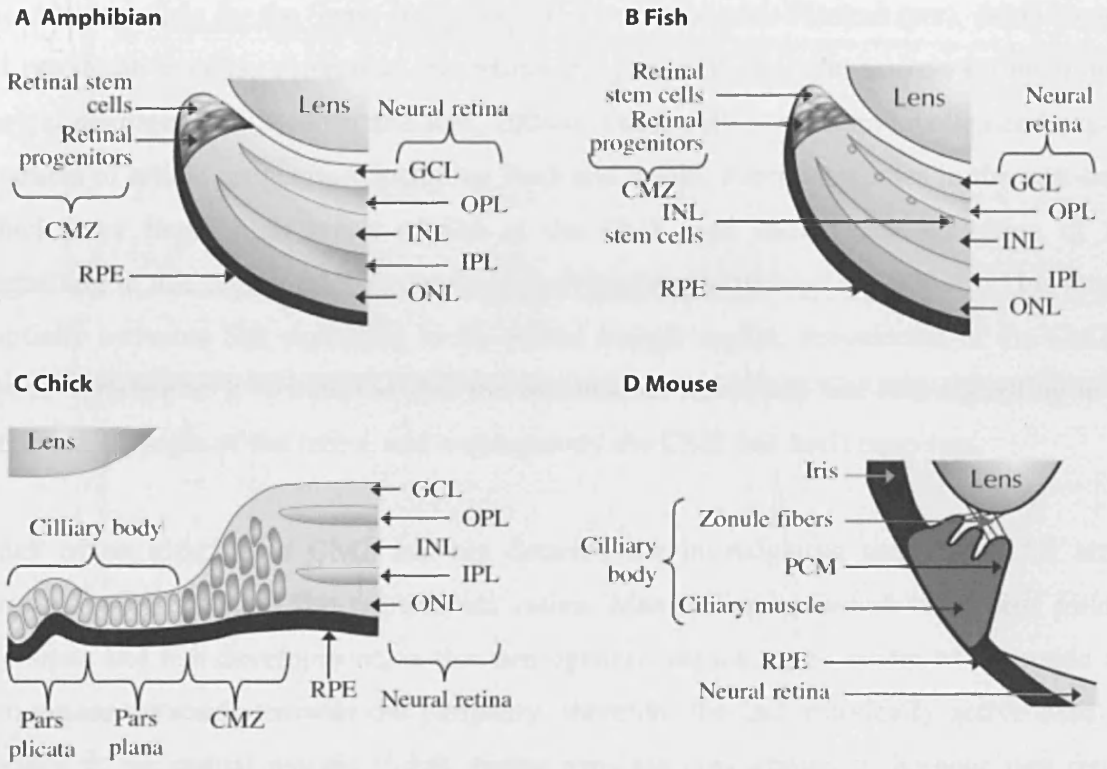

Figure 1.5 Localisation of retinal stem cells in various vertebrates
Schema illustrating the position of stem cells in (A) Xenopus, (B) Fish, (C) post hatched chick and (D) adult mouse retinae. Multipotent retinal stem cells are found in the most peripheral region of the ciliary marginal zone (CMZ) of Xenopus, zebrafish and chick retinae. In the adult mouse retina, cells with stem cell properties (progenitor cells) are found in the pigmented ciliary epithelium (PCM). GCL: ganglion cell layer; INL: inner nuclear layer; IPL: inner plexiform layer; OPL: outer plexiform layer; RPE: retinal pigment epithelium. (Adapted from Amato *et al.*, 2004)

1.6.2 Evidence of neural stem cells in mammals

The CMZ is a specialized region for the generation of retinal neurons in Xenopus, fish and chick, but subsequent searches for a corresponding region in the mammalian retina have been elusive. Studies in mouse mutants have nevertheless provided evidence that mammalian retinae may be capable of regeneration, thus mouse mutants with a single functioning allele for the Sonic Hedgehog (Shh) receptor gene Patched (ptc), exhibit a zone of proliferative cells, situated at the retinal margin, well after the normal termination of retinal neurogenesis (Moshiri and Reh, 2004a). These cells have been labelled and express markers of retinal progenitors including Pax6 and nestin. Previous studies in the post-hatch chick have found high levels of Shh at the CMZ, and shown that inhibition of Shh signalling in this region inhibits neuronal proliferation (Moshiri *et al.*, 2004b). This mutant partially activates Shh signalling in the retinal margin region, reminiscent of the CMZ in lower vertebrates. It is believed that the mammalian retina has lost Shh signalling at the peripheral margin of the retina, and consequently the CMZ has itself been lost.

Lack of an identifiable CMZ has not deterred the investigation and search for active progenitor cells within the mammalian retina. Mammalian retinal development follows Xenopus and fish development, in that neurogenesis begins in the centre of the retina and progresses outwards towards the periphery, therefore the last mitotically active cells are located at the retinal margin. Adult mouse eyes are now known to harbour rare retinal progenitor cells that reside in the pigment epithelium of the ciliary body, a region that lies in between the ciliary marginal zone, and the iris and retina (Amato *et al.*, 2004). These cells exhibit certain qualities of stem cells such as the ability to proliferate, self-renew and express markers of undifferentiated retinal progenitor cells (Chx10 and nestin). Another key finding has been the ability of these cells to differentiate into different retinal types including bipolar cells, müller glial cells and rod photoreceptors. However, differentiation of ganglion, horizontal and amacrine cells has not been observed in these studies (Ahmad *et al.*, 2000; Tropepe *et al.*, 2000). These findings demonstrate the presence of cells with retinal stem cell characteristics in the pigmented epithelium of the ciliary body, and the results have led to an explosion of new data identifying retinal stem cells/progenitor cells in mammalian retinae at various time points from embryonic to foetal development (Ahmad *et al.*, 1999; Yang *et al.*, 2002a).

1.6.3 Retinal pigment epithelium and its regenerative ability

Another source of retinal regeneration, in amphibians, is the RPE, which has been proven to generate new retina when transplanted ectopically into the vitreous of another eye (Stone and Steinitz, 1957). RPE transdifferentiation into neural retina has also been observed in the chick eye when the neural retina has first been removed (Pittack *et al.*, 1991). Several signalling pathways have been implicated in the transdifferentation process, e.g., fibroblast growth factors cause the transdifferentation of the retinal pigment epithelium (RPE) into the neural retina (Park and Hollenberg, 1989).

1.7 Stem cell types

There are three sources from which human retinal progenitor cells could be extracted, each with its own advantages and disadvantages. The use of embryonic stem cells, extracted from the blastocyst, could be a viable source. The cells would be pluripotent, so would hold the greatest level of plasticity of the three; however this may also be a disadvantage, as more cues would be required to differentiate these cells to the final photoreceptor phenotype. As a second option, foetal stem cells would be partially differentiated so fewer steps should theoretically be necessary. Compared to their adult counterparts, the plasticity of foetal stem cells presents a greater hope of successful differentiation. The differentiation potential of adult stem cells is assumed to be limited, however a large body of evidence now supports the idea that certain adult stem cells, particularly bone marrow, can engraft alternative locations that are non-haematopoietic in origin. However the field is not without its detractors.

1.7.1 Potential of human foetal tissue

The use of foetal cells and tissue has led to some encouraging results. Of relevance to this study, several groups have found that human retina from 10-13 week old foetuses harbours progenitor cells that can be maintained and expanded in culture (Yang *et al.*, 2002). Further analysis of human foetal retinal cultures have proven their ability to respond to growth factors and hormones and differentiate towards a photoreceptor lineage (Kelley *et al.*, 1995).

1.7.2 Primary cultures versus immortalised cell lines

As development progresses, progenitor cells become an increasingly finite population. The use of such cells in a clinical capacity is limited because of the difficulty in obtaining sufficient numbers, and the difficulty of maintaining these cells *in vitro*. Additionally, the potential of the cells to replicate *in vitro* may be limited, and cultures of primary progenitor cells frequently comprise a heterogeneous cell population that are at various developmental time points. Therefore it is harder to obtain a homogenous population, and in the vast numbers required. The factors controlling vertebrate retinal progenitor cells would be more accessible to molecular analysis if cell lines with characteristics of the 'parental' cells were established (Frederiksen *et al.*, 1988). Immortalised stem cells have already been used in attempts to repair damage in the brain, and important issues raised from such studies show the need for the immortalising gene to be under the control of a regulatable factor, ideally one that results in the cell line becoming conditionally immortalised.

The most widely used example of this is the temperature sensitive (ts) mutated allele (A58) of the simian virus 40 (SV40) large T antigen (T) oncogene, which is temperature-sensitive due to the presence of two point mutations on the tsT gene (Davies *et al.*, 2003). This is stably expressed in cells cultured at 33°C but is down-regulated at normal body temperature of 37°C. SV40 is a DNA virus that kills and lyses simian cells, its natural host (Cepko, 1989). The early region genes of the virus are capable of disturbing cell cycle pathways, specifically interfering with cell cycle inhibitor genes. This causes loss of cell senescence, leading to the immortalisation of the transfected cell. The actual vehicle for immortalisation is the large T antigen, which disables two tumour suppressor genes, one being p53, which functions as a transcription factor (Carnero *et al.*, 2000) that normally restrains proliferation by inducing the expression of genes which lead to growth arrest. The second target of SV40 is the retinoblastoma gene (Rb), a tumour-suppressor that regulates a cell cycle checkpoint. Following transfection of the SV40 virus into the cell, the tsT protein binds to a domain on Rb that leads to the dissociation of Rb cellular factors that regulate cell cycle progression.

Neural transplantation using immortalised stem cells or progenitor cells has shown significant promise, and there has been no evidence of tumour formation or gross disruption of host brain tissue after grafting (Gray *et al.*, 1999). Transplantations into the developing

rat brain have shown integration and differentiation into both neurons and glia, using tsT-SV40 immortalisation in hippocampal cells (Renfranz *et al.*, 1991). Alternatively the oncogene v-myc which can be used to infect human embryonic brain cells, may be conditionally regulated with tetracycline. The removal of tetracycline switches on v-myc which results in the differentiation of some cell lines towards neuronal and glial lineages (Sah *et al.*, 1997). With specific reference to the work undertaken in this thesis, other groups have shown the ability of immortalised human foetal retinal cell lines to undergo neuronal differentiation with a subset of cells expressing opsin protein (Dutt *et al.*, 1994). However, this study did not use a conditionally immortalised SV40 antigen. Therefore, it will be interesting to examine whether differentiation occurs when the SV40 oncogene is 'switched off' in this study.

1.8 Aims of the thesis

The focus of this thesis is to characterise clonally immortalised human foetal retinal progenitor cell lines, generated by ReNeuron from 10-13 week aborted foetuses, and to ascertain their potential to differentiate *in vitro*, with particular emphasis towards rod photoreceptor differentiation. In doing so, the aim is to determine the developmental cues required to drive these cells towards retinal cell-specific lineages. A secondary goal is to transplant these cells into developing and diseased rat retinae in order to assay their potential to survive, integrate and differentiate *in vivo*. The fundamental aim of this research is therefore to establish whether immortalised progenitor cells could play a role in future clinical trials for cellular therapy, and perhaps alleviate the blindness caused by retinal dystrophies.

Chapter 2

Materials and Methods

Chapter 2

Materials and Methods

2.1 Generation of immortalised cell lines

2.1.1 Derivation of human retinal progenitor cell lines

Two foetal human retinal progenitor cell lines were derived from 10-13 week old gestation foetal eye tissue that was obtained from the MRC Tissue Bank, Hammersmith Hospital, London in accordance with national, legal and ethical guidelines. The following cell line derivation of the progenitor cells was carried out by ReNeuron. Tissue was collected in ice-cold HBSS without Ca^{2+}/Mg^{2+} (Gibco), containing 1 mM N-acetyl cysteine (Sigma). The neural retina was removed from the eyes and cleaned of debris under a dissection microscope (Leica). The tissue was dissociated by first dicing with a scalpel followed by incubating in 0.25% w/v trypsin (Cambrex Biosciences, Belgium) in DMEM:F12 (Gibco) containing 0.025 U/ml Benzonase (Merck) for 15 min at 37°C. Following dissociation, twice the volume of trypsin inhibitor solution (5.5 mg/ml soybean trypsin inhibitor, Sigma, dissolved in DMEM:F12, containing 1% human serum albumin, Grifols, and 0.025 U/ml Benzonase) was added. Cells were centrifuged at 800 x g for 5 min, then resuspended in serum-free human media comprising DMEM:F12 supplemented with human serum albumin (0.03%); human transferrin, (100 µg/ml, Sigma); putrescine dihydrochloride (16.2 µg/ml Sigma); human insulin (5 µg/ml, Sigma); L-thyroxine (T4) (400 ng/ml, Sigma); tri-iodo-thyronine (T3) (337 ng/ml, Sigma); progestrone (60 ng/ml, Sigma); L-Glutamine (2 mM, Gibco); sodium selenite (selenium) (40 ng/ml, Sigma); heparin sodium salt (10 U/ml, Sigma); corticosterone (40 ng/ml, Sigma) and Gentamycin (50 µg/ml, Gibco) in the presence of basic fibroblast growth factor, bFGF, (10 ng/ml, Invitrogen) and epidermal growth factor, EGF (20 ng/ml, Sigma). The yield of cells isolated from the neural retina of two eyes was approximately $3x10^6$, with 90% viability as judged by trypan blue exclusion.

Cells were plated in 2 wells of a 6-well plate (Falcon) coated with laminin (Sigma, coated at 5 µg/ml in DMEM:F12 for 1 h at 37°C followed by one wash with DMEM:F12) and maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Upon reaching confluency cells were passaged to laminin-coated flasks using trypsin and trypsin inhibitor solutions, detailed above. Following two passages (P3) cells were infected at 50%

confluency for 8 h with fresh media containing retrovirus carrying the temperature-sensitive TsT-SV40 antigen gene (pLNCX-SV40-U19tsA58) in the presence of 4 μg/ml polybrene. Three successive 8 h infections were carried out. Following infection, cells were placed under antibiotic selection (150 μg/ml Geneticin, Gibco). Clonal isolation was carried out using the cloning ring method by plating 2,000-10,000 cells in a 15 cm laminin-coated round tissue culture dish and picking isolated colonies after 2 to 3 weeks growth using 8 x 8 mm cloning rings (Sigma). A total of 11 clones were isolated, expanded and frozen down in 10% DMSO (Wak-Chemie), human medium in the presence of bFGF and EGF. Two stable clonal cell lines were used in this study, namely GuRt09 and GuRt05. Cells were routinely cultured at the permissive temperature of 33°C, at which temperature the cells proliferate but do not differentiate. To induce differentiation the cells were transferred to the non-permissive temperature of 37°C.

2.2 Cell culture

2.2.1 Retinal progenitor cell culture

Unless otherwise stated, cells were cultured in T75 tissue culture flasks (Nunc, UK) and incubated at 33°C in water-saturated air with 5% CO_2. Human foetal retinal progenitor cell lines GuRt09 and GuRt05 were obtained from ReNeuron (Guildford, UK) as described above in section 2.1.1 and were incubated at 33°C in DMEM:F12 media minus L-Glutamine (Gibco) supplemented with 0.03% (v/v) human serum albumin (Grifols, UK), 100 μg/ml human transferrin, 16.2 μg/ml dihydrochloride putrescine, 5 μg/ml human recombinant Insulin, 400 ng/ml L-Thyroxine (T4), 337 ng/ml Tri-Iodo-Thyronine (T3), 60 ng/ml progesterone, 2 mM L-Glutamine, 40 ng/ml sodium selenite, 10 units/ml heparin, 40 ng/ml corticosterone and 50 μg/ml Gentamycin (Gibco). Cells were provided with fresh medium twice weekly with 10 ng/ml bFGF (Peprotech) and 20 ng/ml EGF (Peprotech) along with a full media change. In addition to the growth factors the culture flasks were coated with laminin (Sigma). Laminin was dissolved in cold DMEM:F12 media minus L-Glutamine and applied to the flasks at a coating concentration of 1-5 μg/cm^2. The flasks were incubated overnight at 37°C and washed with warmed medium the following day. The cells were passaged once a week or when required. For passaging, cells were washed once in PBS without Ca^{2+}/Mg^{2+} (Gibco) and incubated with trypsin (2.5 ml for a T75 and 1 ml for a T25) (Cambrex Biosciences, Belgium) at 37°C until the cells detached. Trypsin was

inactivated with a soybean trypsin inhibitor by dilution with double the quantity used for trypsin. The cells were then pelleted by centrifugation at 12000 x g for 5 minutes. Cells were usually seeded at a density of 0.5 x 10^6 cells per T25 and 1.0 x 10^6 cells per T75. For all experiments cells were seeded at a density of 10,000 cells/ cm^2 and either placed in a 33°C or 37°C incubator as required.

The unimmortalised GS076 cells were cultured in the same way as outlined above with three main differences. Culture flasks were coated with fibronectin at a coating concentration of 1-5 $\mu g/cm^2$ as opposed to laminin, and the human medium was supplemented with 5% Foetal Bovine Serum (FBS) in addition to all the above components. The cells were incubated at 37°C, as opposed to 33°C, in water-saturated air with 5% CO_2.

2.2.2 Culture of other cell lines

All other cell types used in this thesis were cultured in T25 or T75 tissue culture flasks (Nunc, UK) and incubated at 37°C in water-saturated air with 5% CO_2. The ARPE19 cell line were maintained in DMEM:F12 media plus L-GlutaMAX™ (Invitrogen) supplemented with 10% (v/v) Foetal Bovine Serum (FBS) and 1.0% (v/v) Penicillin/Streptomycin (Gibco). For passaging, ARPE19 cells were washed with PBS (Gibco) and incubated with 1 ml trypsin at 37°C until the cells detached. Trypsin was inactivated by dilution with 2 ml of full DMEM:F12 medium and the cells pelleted at 12000 x g. Cells were split 1:5 once a week and fed twice weekly.

2.2.3 Determination of cell number

The number of viable cells was assessed by diluting 10 µl of cell suspension in culture medium with 10 µl of a 0.4% trypan blue solution (Sigma). The cell mixture was left for 2-3 min at room temperature after which an aliquot was placed into a haemocytometer. Cells were examined under a microscope and the number of viable, unstained cells within the 1 mm x 1 mm x 0.1 mm grid counted. This number was multiplied by 1 x 10^4 and the dilution factor to give the number of million cells per ml.

2.2.4 Cryopreservation

Detached cells were centrifuged at 12000 x g for 5 min and resuspended in a freezing solution comprising 0.2 µm filter-sterilised complete human growth medium and 10% (v/v)

dimethylsulfoxide (DMSO), at a final cell density of approximately 1 x 10^6 cells/ml. The suspension was quickly dispensed in 1 ml aliquots into labelled cryovials (Nunc, UK). The vials were held at -80°C for 24 h in a polystyrene box, and then transferred to liquid nitrogen for long term storage. To thaw, cells were warmed quickly in a 37°C water bath, diluted in their respective growth media, pelleted by centrifugation and resuspended in the appropriate volume of media into a T25 flask. Fresh medium was applied to the cells the following day.

2.2.5 Treatment with all-*trans*-retinoic acid

Stock solutions of 10 mM all-*trans*-retinoic acid (RA) (Sigma) were prepared in low light by reconstituting in dimethylsulfoxide (DMSO) and stored in foil-wrapped 50 µl aliquots at -80°C. Human foetal immortalised retinal progenitor cells were seeded onto a series of laminin coated 35-mm tissue culture dishes at a density of 1.0 x 10^4 cells/cm^2. The standard cell culture medium of DMEM:F12, minus L-Glutamine was supplemented with 50 µg/ml Gentamycin and either 500 nM, 1 µM or 3 µM all-*trans*-RA, which was diluted to the appropriate concentration in the culture medium just prior to use. Due to the light sensitive nature of RA and DMSO used during this experiment, all medium changes were carried out in minimal light. During the culture period, the dishes were covered with aluminium foil. The experiment was performed for a duration of three days with daily medium changes.

2.2.6 Treatment with all-*trans*-retinoic acid in the presence of 3% charcoal/dextran treated FBS

Cells were also cultured with all-*trans*-RA in the presence of 3% charcoal/dextran treated foetal bovine serum (FBS) (Hyclone) as s supplement to the culture medium. Instead of adding the all-*trans*-RA when the cells were seeded, a different methodology was applied as described in a previous study (Chen *et al.*, 2003). Briefly, cells were seeded on culture dishes and grown in human medium with 20 ng/ml EGF and 10 µg/ml bFGF at 37°C. After 1 day the medium was replaced with DMEM:F12 minus L-Glutamine and 3% charcoal/dextran treated FBS. After 24 h, cells were treated with 0.5-3 µM all-*trans*-RA diluted in DMEM:F12, minus L-Glutamine and 3% charcoal/dextran treated FBS. This was considered day 1 of the experiment and daily medium changes were carried out for up to 7 days when the cells were fixed and immunoassayed.

2.3. Reverse-transcription polymerase chain reaction

2.3.1 Isolation of total RNA

Total RNA was isolated using TRIzol reagent (Invitrogen, UK), according to the manufacturer's instructions. Briefly, cells were cultured in a 60 mm culture dish and seeded at a density of 10,000 cells per cm^2 and grown for 7 days or as described in the results sections. The cells were then lysed directly from the culture dish by first removing the spent media and then adding 0.5 ml TRIzol reagent directly on to the cells. A cell scraper was then used to remove the cells and gather a viscous lysate. The viscous cell lysate was triturated several times, transferred to a sterile Eppendorf tube and incubated for 5 min at room temperature.

Next, 0.1 ml chloroform was added for every 0.5 ml of TRIzol reagent used, in order to extract the nucleic acids. The solution was vigorously mixed by hand for 15 s and then allowed to incubate at room temperature for 3 min. Subsequently, the solution was centrifuged at 12000 x *g* for 15 min at 4°C.

The upper, aqueous phase containing the RNA was carefully transferred to a fresh Eppendorf tube and the RNA precipated by adding 0.5 ml of isopropanol for every 1 ml TRIzol reagent added, and 1 µl glycogen (Ambion, TX, USA) to help visualise the eventual pellet. The mixture was briefly mixed, incubated on ice and the RNA pelleted by centrifugation at 12000 x g for 10 min at 4°C. The pellet was washed in 1 ml 75% ethanol for every 1 ml of TRIzol reagent used. The mixture was vortexed to loosen the pellet from the side of the tube and then centrifuged at 7,500 x g for 5 min at 4°C. The resulting supernatant was aspirated and the pellet was allowed to dry at room temperature for 3-4 min. The RNA pellet was resuspended in 16 µl DEPC-treated water and dissolved by incubating at 65°C for 10 min with intermittent vortexing.

2.3.2 Quantification of RNA

RNA was quantified using a spectrophotometer (Eppendorf BioPhotometer). For quantification 1 µl of the RNA sample was diluted with 99 µl DEPC-treated water, and the A_{260nm} reading recorded. The concentration of RNA was calculated using the following equation:

$$\text{RNA concentration } (\mu g/ml) = A_{260nm} \text{ reading} \times 40 \times 100$$

where 40 is the µg/ml RNA equivalent to an A_{260nm} of 1 and 100 is the dilution factor. The concentration of RNA was derived as a mean of three measurements. After quantifying the RNA it was stored at -80°C in 4 µl aliquots.

2.3.3 RNA analysis by agarose formaldehyde gel electrophoresis

Unlike DNA, RNA has a high degree of secondary structure therefore it has to be denatured by treatment with formamide and separated by electrophoresis through agarose gels containing formaldehyde (Sambrook *et al.*, 2001). This method was used to ascertain the quality of RNA extracted using the TRIzol extraction method. Briefly, a 1.2% (w/v) agarose gel was made with RNase free water and boiled in the microwave. The solution was allowed to cool for approximately 30 min at which point the 10 x MOPS electrophoresis buffer (0.2 M MOPS, 20 mM NaAc, 10 mM EDTA, pH 7.0), formaldehyde and ethidium bromide were added. The RNA samples were mixed with RNA loading mix in the ratio of 2:1, denatured at 65°C for 15 min and quenched on ice for a further 2 min. Samples were centrifuged for 5 s before adding 6x loading buffer, loaded onto the gel and resolved at 100 V for 2 h. RNA bands were visualised on a UV light box.

2.3.4 DNase treatment

A commercially available DNase treatment kit (Promega, Madison WI) was used to degrade any remaining double- or single-stranded DNA that could interfere with the subsequent RT-PCR reactions. Briefly, 1-8 µl of RNA was added to a sterile Eppendorf tube, to which 1 µl RQ1 RNase-Free DNase 10 x Reaction Buffer and 1 µl RQ1 RNase-Free DNase I Endonuclease were added. If required the final volume was made up to 10 µl with nuclease-free water and incubated at 37°C for 30 min. In order to terminate the reaction, 1 µl of RQ1 DNase STOP solution was added and incubated at 65°C for 10 min to inactivate the DNase. This 11 µl reaction could then directly be used for cDNA synthesis.

2.3.5 First-strand cDNA synthesis

Single-stranded cDNA was prepared from the extracted total RNA, pre-treated with DNase I Endonuclease using an oligo(dT) primer, and reverse-transcribed using Moloney Murine

Leukemia Virus (M-MLV) Superscript™ III reverse transcriptase (Invitrogen) following the manufacturer's protocol. Briefly, 1 µl oligo $(dT)_{20}$ (50 µM), 1 µl 10 mM dNTP mix and the 11 µl DNase treated total RNA were placed into a nuclease-free Eppendorf microcentrifuge tube and the reaction made up to a total volume of 13 µl, if required, with sterile water. The mixture was heated to 65°C for 5 min and then transferred onto ice before the addition of 4 µl 5 X first strand buffer, 1 µl 0.1 M DTT, 1 µl RNaseOUT™ Recombinant RNase Inhibitor (40 units/µl) and 1 µl Superscript™ III Reverse Transcriptase (200 units/µl). The contents were briefly mixed by gentle pipetting and incubated at 50°C for 50 min before inactivation of the reaction at 70°C for 15 min. Alongside the synthesis of cDNA a control was run simultaneously from the same RNA sample that was treated in exactly the same way minus the addition of Superscript Superscript™ III Reverse Transcriptase. Throughout this thesis this control is referred to as a "no RT" control. The cDNA template was used in a standard PCR using a Mastercycler® Gradient Thermocycler (Eppendorf, UK). The primers (Table 2.1) used were designed against human cDNA sequences or were from previously published papers.

Gene Name	Primer Sequence 5'-3'	Optimal Annealing Temperature (°C)	Amplicon Size	Genbank Accession Number/ Reference
Nestin	F:GAATCACTGAAGTCTGCGGGAC R:TCCAGGAGTCTGAATGTCTCTTGG	61.5	176bp	X65964
NF160	F:TGCTCCCTCCTCAGTCTTTGG R:TCGTTTATTGTTTTTGGCTCAGTTG	61.5	155bp	BC 096757
NF200	F: GCCAAGGTGGAGGTGAAGGA R: TGGTCTGTGCTGGAGGATTTTT	61.5	271bp	NM 021076
Recoverin	F: GGAAAAGCGAGCCGAGAAGA R: CCTGGGGTGGATGTGTGTGT	61.5	282bp	NM 002903
Rhodopsin	F: AACTGCTCCCCCTTCTCCAT R: GCTTGGCTCTGCTCATTGCT	61.5	227bp	NM 000539
β III Tubulin	F:GCGAGATGTACGAAGACGAC R:TTTAGACACTGCTGGCTTCG	56.5	115bp	Mozzetti *et al.*, 2005
Sox2	F:CGCCCCCAGCAGACTTCACA R:CTCCTCTTTTGCACCCCTCCCATTT	59.6	170bp	Tsukamoto *et al.*, 2005
Hes1	F:CAGCCAGTGTCAACACGACAC R:TCGTTCATGCACTCGCTGA	56.3	307bp	Klassen *et al.*, 2004
Ki67	F:TACGTGAACAGGAGCCAGCA R:CAGCTGCATTTCTGCCATTA	58.8	246bp	NM 002417
Nrl	F:GGCTCCACACCTTACAGCTC R:GGCCCATCAACAGGGACTG	60.0	212bp	NM 006177
Crx	F:CCTCAGTGTCCCCGAAGAT R: CTGAACACCGAGCTGTCAGA	60.0	1059bp	NM 000554
S-Opsin	F: TAGCAGGTCTGGTTACAGGATG R: GAGACGCCAATACCAATGGTC	60.0	148bp	Lawrence *et al.*, 2007
GAPDH	F:CTTTGGTATCGTGGAAGGACTC R:TCTTCCTCTTGTGCTCTTGCTG	58.8	550bp	Yu *et al.*, 2004
Human β Actin	F:ACACCTTCTACAATGAGC R:ACGTCACACTTCATGATG	58.8	600bp	Wong *et al.*, 2004
GAPDH	F:TGCACCACCAACTGCTTAG R:GGATGCAGGGATGATGTTC	60.0	200bp	XR 015759

Table 2.1 List of primers used throughout this study
Primers were used at the specified annealing temperatures to amplify markers in unimmortalised and immortalised human foetal retinal progenitor cells and the cDNA from a human retinal library as a positive control.

2.3.6 Polymerase Chain Reaction (PCR)

PCRs were carried out using Go Taq Flexi Kit (Promega), with amplification conditions as follows: 95°C for 5 min followed by 35-40 cycles of 94°C for 1 min, annealing temperature appropriate to primer pair (Table 2.1) for 1 min and 72°C extension for 1 min, followed by a final extension at 72°C for 4 min. PCRs of retinal progenitor cell cDNA were carried out alongside both water (no-template control) as well as no-reverse transcriptase (no RT) controls. Prior to use on retinal progenitor cDNA, the specificity of all primers was tested on a phage (λ GT10) human retinal cDNA library (a kind gift from Dr A. Hardcastle).

PCR reaction

0.5 µl cDNA
0.5 µl primer forward (10 pmol/µl)
0.5 µl primer reverse (10 pmol/µl)
2.5 µl *Taq* reaction buffer
0.5 µl 10 mM dNTPs
2.0 µl-2.5 µl 25 mM $MgCl_2$ (dependent on primer pair)
2 Units *Taq* polymerase
Sterile ddH_2O to 25 µl

2.3.7 Agarose gel electrophoresis

PCR products were separated and analysed by horizontal agarose gel electrophoresis, as described elsewhere (Sambrook *et al.*, 2001). DNA samples were routinely resolved on a 2% (w/v) agarose gel prepared by adding 3 g of agarose to 150 ml 1 x Tris-acetated-EDTA (TAE) buffer (2 mM Tris-HCl, pH 8.0, 50 mM EDTA, pH 8.0, 5.7% acetic acid) and dissolved by boiling in a microwave and cooled to approximately 50°C. Ethidium Bromide (0.5 µg/ml) was added to the gel solution before being cast. PCR products were prepared by the addition of DNA loading buffer (80% glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol FF). The gel was electrophoresed at 100 V for 1-2 h or until the DNA fragments were sufficiently resolved, appropriately sized DNA markers (New England Biolabs, UK) were also electrophoresed on the gel to determine sample fragment size. The ethidium

bromide stained gel was visualised using an UV transilluminator and images obtained using Gene Snap (Syngene).

2.4. Immunocytochemistry

Primary antibodies used, optimal dilutions and source are shown in table 2.2. Cells were cultured in 35 mm dishes at a density of 10,000 cells per cm^2 and grown for 1 week in the various conditions. Spent medium was aspirated and cells fixed immediately with 4% paraformaldehyde for 30 min at 4°C. Following several washes with 0.1 M PBS pH 7.4 (10 x PBS; 80.9g NaCl, 2.9 g KCl, 2.9 g KH_2PO_4, 11.5g Na_2HPO_4, pH 7.3) cells were incubated in a series of solutions, all made up in 0.1 M PBS containing 0.3% Triton X-100 (Sigma). Cells were permeabilised in 0.3% Triton X-100 (Sigma) in 0.1 M PBS for 2 min at room temperature. After permeabilisation, cells were washed with 0.1 M PBS three times for 5 min each, and then blocked in 5% normal donkey serum (NDS) (Jackson Immunoresearch) for 30 min at room temperature. Samples were then incubated with the primary antibody, or combination of antibodies, made up at the appropriate dilution (table 2.2) in the presence of 1% NDS at 4°C overnight. The following day, after three successive washes with 0.1 M PBS the cells were incubated in the secondary antibody, diluted at 1:200 with 2% (v/v) blocking solution for 30 min, in the dark. Following the removal of the secondary antibody solution, at least 4 washes were performed to rinse the cells, to minimise background staining. After washing, excess moisture was removed from the edge of the dish with a tissue, and a drop of Vectashield mounting media containing DAPI [nuclear stain] (Vector Laboratories, Peterborough UK) was placed on to the centre of the dish. A coverslip was placed on top of the mounting media; care was taken to avoid the formation of air bubbles. To secure the coverslip in place, nail varnish was applied around the edges and allowed to air dry.

Target Protein	Reactivity	Optimal Dilution	Catalogue No. & Source	Target cells/ other features identified
Nestin	Goat Polyclonal	1:200	sc-21247 Santa Cruz	Neural Stem Cells
NF160	Mouse Monoclonal	1:1000	MAB5254 Chemicon	Retinal Ganglion Cells/ Horizontal Cells
NF200	Rabbit Polyclonal	1:5000	AB1982 Chemicon	Retinal Ganglion Cells/ Horizontal Cells
Recoverin	Rabbit Polyclonal	1:1000	AB5585 Chemicon	Rods, Cones and Bipolar Cells
Rhodopsin	Rabbit Polyclonal	1:200	sc-15382 Santa Cruz	Rods
β III Tubulin	Mouse Monoclonal	1:1000	MAB1637 Chemicon	Retinal Ganglion Cells
Sox2	Goat Polyclonal	1:500	sc-17319 Santa Cruz	Retinal Progenitor Cells
Nrl	Rabbit Polyclonal	1:500	sc-33183 Santa Cruz	TF involved in rod photoreceptor differentiation
Crx	Goat Polyclonal	1:500	sc-22381 Santa Cruz	TF involved in rod photoreceptor differentiation
Rhodopsin Kinase	Mouse Monoclonal	1:1000	R 3276 Sigma	Rod and cone photoreceptors
S-Opsin	Goat Polyclonal	1:500	sc-14363 Santa Cruz	Blue light sensitive cones
Chx10	Goat Polyclonal	1:500	sc-21690 Santa Cruz	Eye Field TF and bipolar cells
GFAP	Mouse Monoclonal	1:500	G 3893 Sigma	Astrocytes and other glial cells
Ki67	Rabbit Polyclonal	1:2000	NCL-Ki67p Novocastra Laboratories	Active phase of cell cycle
Human Mitochondria	Mouse Monoclonal	1:1000	MAB1273 Chemicon	Human Mitochondria

Table 2.2 List of primary antibodies used throughout this study
All antibodies were used at the specified dilution in PBS and 0.3% Triton X-100 with 1% normal donkey serum, unless otherwise stated. TF: Transcription Factor.

2.4.1 Confocal microscopy

Fluorescently stained cells were examined and images captured using a Zeiss confocal laser scanning microscope with LSM 510 software. Images in figures are stacked confocal images unless otherwise stated.

2.5 Subretinal transplantation surgery

All animal care was in accordance with Institutional and Home Office (UK) regulations and the UK Animals (Scientific Procedures) Act, 1986. For the transplantation procedure rats were anaesthetised with intraperitoneal injections of Ketamine (5 mg/kg) and Xylazine (10 mg/kg) and the pupil was dilated using Tropicamide (1% Mydriacyl®, Alcon Labs., Hemel Hempstead, UK). Prior to transplantation, cultures of the immortalised cells were trypsinized, washed and counted into a suspension of approximately 5 x 10^4 cells/µl in DMEM:F12 minus L-Glutamine (Gibco). Cell suspensions were stored on ice prior to the transplantation procedure.

The grafting of cells into the eye was visualised using an operating microscope (Olympus). Cells were injected through a fine glass pipette (internal diameter 200 µm) attached to a 10 µl Hamilton syringe. Following the back-filling of injection equipment with carrier medium (DMEM), a small opening was made in the eyelids using fine microsurgical scissors. Then, using a trans-scleral approach, either sham or cell suspension injections, approximately 1-2 µl per eye, were made unilaterally into the vitreous chamber or subretinally of the left eye. Upon completion of surgery, the animals were given a second intraperitoneal injection consisting of the anaesthetic antidote Antisedan® (atipamezole hydrochloride 5 mg/kg). Once fully conscious, pups were returned to the home cage. All animals were killed at various time points post-transplantation either 5 or 26 days post graft or 9 weeks post graft. Animals were placed on *ad libitum* oral cyclosporine A (concentrate infusion, Sandimmun, Sandoz, Camberley, UK; 210mg/l of drinking water) from 2 days before transplantation until killing to limit the possibility of immune rejection of the grafted cells.

2.5.1 Tissue extraction and preparation

Animals were deeply anaesthetised with Pentobarbital Sodium (200 mg/kg) and perfused transcardially with 0.1 M PBS, followed by 4% paraformaldehyde. Eyes were removed, cornea punctured and orientation noted before placement in the same fixative overnight at

4°C. The following day eyes were washed in PBS and cryoprotected in 30% sucrose solution (in PBS). Following one further day in sucrose at 4°C the lens was carefully removed from each eye, which was orientated prior to freezing. The eyecup was then embedded in Optimal Cutting Temperature Compound (OCT;Tissue Tek®, Miles, Raymond Lamb, London, UK), rapidly frozen in a freezing mixture of dry ice and acetone and stored at -70°C. Frozen blocks were sectioned (12 μm-14 μm) using a Leica CM3050 Cryostat and mounted on charged glass slides (BDH) in 5 consecutive series. The slides were allowed to air dry for 2 hours and stored at -80°C and used for immunohistochemical studies. Prior to staining, frozen sections were brought to room temperature and dried in a current of air for one hour.

2.5.2 Immunohistochemistry

Slides were placed in a humidity chamber and blocked with for 1 h with 5% NDS in 1x PBS (+ 0.3% Triton-X100). Primary antibodies were prepared in PBS containing 0.3% Triton X-100 with 1% NDS, and 500 μl was incubated on the slides overnight. The following day the primary antibody solution was washed off with 3 x 10 min washes in PBS. Secondary antibodies were made up in PBS (plus 0.3% Triton X-100) with 2% NDS and applied to tissue for 2 h. Once the secondary antibody was removed cell nuclei were counterstained with DAPI (1:5000 dilution; Sigma). Slides were washed once in PBS for a duration of 5 min, and then extensively in Tris buffer (0.05 M pH 7.4), prior to being cover-slipped with vectashield (Vector labs) and secured with nail varnish.

2.5.3 Immunolabelling of retinal sections to distinguish xenografted human cells from host macrophage/microglia

Previous experience has shown that host macrophages/microglia is responsible for an autofluorescent staining artefact that may lead to false positive identification of grafted cells (A.Vugler, personal communication). In order to control for this and to unambiguously identify grafted human cells a protocol visualising the ED1 antibody (specific for CD68 on rat macrophages and microglia) with peroxidase was employed prior to labelling other relevant antibodies with fluorescent markers. After blocking with 5% normal horse serum (NHS) in 0.1 M PBS with 0.3% Triton X-100 sections were incubated in mouse anti-ED1 antibody in PBS (plus 0.3% Triton X-100) with 1% NHS overnight at

room temperature. After washing in PBS, sections were incubated for 2 h in PBS-TX containing biotinylated horse anti-mouse secondary antibody (Vector, 1:200), washed again and incubated for 1 h in a 1:50 dilution of Avidin-Biotin-Peroxidase Complex (Vector, ABC Elite kit) in PBS plus 0.3% Triton X-100. Following a PBS wash, sections were reacted in a 0.05% solution of 3,3'-diaminobenzidine (DAB, Sigma) with nickel and cobaltous chloride added for signal intensification. Slides were incubated for 30 s in this solution containing 0.03% hydrogen peroxide. The peroxidase reaction was terminated by extensive washing. Once treated in this manner the mouse anti-ED1 antibody is not detectable by fluorescently tagged anti-mouse secondary antibodies. Slides were then blocked with 5% normal donkey serum (NDS, Jackson ImmunoResearch) in PBS for 1 h followed by overnight application of the relevant combination of primary antibodies (Table 2.2) as detailed above.

2.6 Flow cytometry

2.6.1 Background

Flow Cytometry is a method that allows the quantification of the physical and chemical characteristics of cells whilst passing through a fluid stream (Weaver, 2000). Therefore, a heterogeneous population composed of thousands of cells can be characterised with respect to cellular characteristics (Patkar *et al.*, 2002). A light beam intersects the fluid stream at a point where a number of fluorescence and other detectors are positioned in order to obtain readings for forward scatter (FSC) and side scatter (SSC) fluorescence. As the cell passes through the light beam it scatters the light to some degree, additionally the fluorescent molecules labelling the cells are excited into emitting light. The light and scattering information are collected and converted to digital values that reveal certain characteristics of that individual cell.

A flow cytometer can measure several parameters simultaneously, these include forward scattered light (FSC) which is proportional to cell size, and side scattered light (SSC) which is proportional to cell granularity. Fluorescence intensities are measured at different wavelengths simultaneously for each cell. The primary goal of flow cytometry is to obtain data that quantify the percentage of cells expressing markers under investigation. Flow Cytometric measurements were performed with a BD FACS Calibur, 488nm argon ion

laser (BD Biosciences). Ten thousand events were collected per sample and stored for analysis. Two colour live gating was used to optimize for each fluorochrome. Data analysis was carried out using WinList, Verity software.

2.6.2 Fluorescent stains

Fluorescence intensities were measured at different wavelengths simultaneously for each cell. The argon ion laser is commonly used in flow cytometry because at 488nm it can excite more than one fluorochrome. The combination of fluorescein isothiocyanate (FITC) and phycoerythrin (PE) were used in these experiments as both are excited at 488nm, and yield fluorescence emission spectra with peak emission wavelengths that are easily resolved.

2.6.3 Gating

Gating defines the cells of interest based upon the physical characteristics of a cell determined by the FSC and SSC signals. This allows the exclusion of dead cells and cell debris from the analysis. Gates are drawn in the dot plot as a polygon encircling the population of interest (Nunez, 2001). Cells within the boundary are analysed for expression of protein markers under investigation.

2.6.4 Quadrant setting

Quadrant settings were established by running a negative control cell sample in which the primary antibody is omitted to indicate the non-specific binding of the secondary antibodies used. The quadrants were set such that 98-99% of all events were included into the lower left quadrant representing cells that bind neither antibody. Subsequently, these quadrants were used for all further analysis of the cells, on that day, with no alterations. A negative control was run prior to each experiment in order to eliminate variability.

2.6.5 Immunocytochemistry on cells in suspension

Briefly, cells in the monolayer culture were trypsinized and counted so that just over 1 x 10^6 cells were aliquoted into individual sterile Eppendorf tubes. The cells were centrifuged at 4,000 x g for 5 min and then fixed with 100 µl cold 4% paraformaldehyde and left at 4°C for 30 min. After further centrifugation, the fixative was removed and the cells were washed once in 100 µl of PBS. Due to the nature of immunostaining cells in suspension,

between each step cells were centrifuged at 4,000 x g for 5 min each. Following a further round of centrifugation cells were permeablised with 0.3% Triton X-100 for 2 min and then washed with PBS. Prior to antibody incubation, cells were blocked for non-specific binding in PBS containing 5% Normal Donkey Serum (Jackson Laboratories), 0.3% Triton X-100 for 30 min at room temperature. Cells were then centrifuged and incubated with appropriate dilutions of antibodies (Table 2.2) and left overnight at 4°C. The following day the cells were centrifuged and then washed once with PBS. The secondary antibody solution was composed of 2% NDS diluted in 0.3% Triton X-100 in PBS and the appropriate dilution of secondary antibody. The cells were incubated in the secondary antibody solution for 30 min then foil wrapped and left at room temperature. Following this incubation cells were centrifuged and then washed one last time before a final centrifugation. The cells were then resuspended into 300 µl of PBS and kept in the dark at 4°C until run on the flow cytometer.

2.7 Use of standard error of the mean

The standard deviation (SD) is a useful estimate of the dispersion of samples, whilst the standard error of the mean (SEM) is used in inferential statistics to estimate the SD of the means of a population. The main aim of this thesis has been to compare and contrast the effects of differently treated cell populations. In order to make a statistical comparison between both groups the calculation of the mean and estimation of its variability (SEM) are required for each sample set. In order to visually illustrate this comparison, data has been plotted on graphs with error bars that represent the SEM throughout this thesis. Therefore, I believe that the use of the SEM, as a measure of dispersion of the mean of each group, would be more appropriate than the SD of the samples in this case.

Chapter 3

Characterisation of immortalised and unimmortalised human foetal retinal progenitor cells

Chapter 3

Characterisation of immortalised and unimmortalised human foetal retinal progenitor cells

3.1 Introduction

Retinal development is a highly specialised process that is conserved spatially and temporally (Ahmad *et al.*, 1999). There exists a basic understanding of the mechanisms controlling retinal histogenesis, however much remains to be elucidated. There are two principle aims of work on retinal development, one being to enhance our understanding of the mechanisms involved in retinal developmental, and the other to harness this knowledge to explore the use of such cells in the application of cellular therapy. As therapeutic tools, these cells may replace and restore lost cell types in various retinal diseases.

Experiments using human retinal progenitor cells from 10-13 week old foetal neural tissue have shown their potential, in the correct microenvironment, to differentiate towards neuroretinal cell types (Kelley *et al.*, 1995; Yang *et al.*, 2002a). While effective in increasing our understanding of retinal development, these cells have a limited application clinically, given the vast numbers of patients that could benefit from such a therapy and the finite number of cells available at present.

The aim of this thesis was to characterise and evaluate the differentiation potential of two human foetal progenitor cell lines that have undergone immortalisation. Immortalised cells escape the controls of the cell cycle that would otherwise constrain proliferation and therefore grow and divide continuously, usually well beyond the limits of primary cells (Stacey and MacDonald, 2001). In this study a temperature-sensitive mutant of the SV40 large T antigen was used for immortalisation, which enables the cells to proliferate at the permissive temperature of 33°C and in principle differentiate at the non-permissive temperature of 37°C. SV40 is a DNA virus that kills and lyses simian cells, its normal host, however when in cells from other species it may integrate and express large T antigen, an immortalising oncogene (Cepko, 1989).

By investigating the potential of, and characterising these SV40 immortalised retinal progenitor cell lines, it was hypothesised that the cells would the express critical characteristics and antigens of their unimmortalised counterparts, and have the potential

to respond to environmental cues by differentiating towards a photoreceptor or other retinal cell lineage. If immortalisation was shown to be compatible with the cells retaining their ability to differentiate, this would lead to vast numbers of clonal cells being generated that could provide new understanding of the mechanisms involved in retinal histogenesis and perhaps be the basis of immortalised cells (using techniques that do not rely on oncogene introduction) being used in the clinics.

To fully understand the development and differentiation of these cells *in vitro* a variety of markers were investigated to identify the developmental stage that the cells exhibit. It was also important to understand what a 10-13 week old foetal retina expresses at this developmental stage (figure 3.1) in order to properly evaluate the expression patterns of the cells *in vitro*. Another important step was to run a comparative study between unimmortalised cells of the same age as those that have been immortalised, to identify differences and similarities between both cell types.

3.1.1 Immunocytochemical and PCR markers

Much of the work in this thesis uses a number of markers to evaluate the developmental and lineage-specific characteristics of these cells, and here a brief overview will be given as to what they are and why they were used.

3.1.1.1 Nestin

Nestin is an intermediate filament protein expressed in mitotically active central nervous system progenitor cells (figure 3.1 A) during early stages of development (Cattaneo and McKay, 1990; Hockfield and McKay, 1985). It is first expressed in the developing rodent neural tube at E11 after the neural tube has closed, when 98-100% of these cells are nestin positive. After only two days some cells are already nestin negative in regions where post-mitotic neurons are known to reside (Hockfield and McKay, 1985). An important feature of nestin as a marker, is that it discriminates between stem cells and more differentiated cells in the neural tube (Lendahl *et al.*, 1990). Nestin mRNA is known to be down-regulated at the end of neurogenesis (Lendahl *et al.*, 1990) and replaced by tissue specific filament proteins (Zimmerman *et al.*, 1994). This gene has thus helped the study of early developmental processes by the identification of specific progenitor cell types.

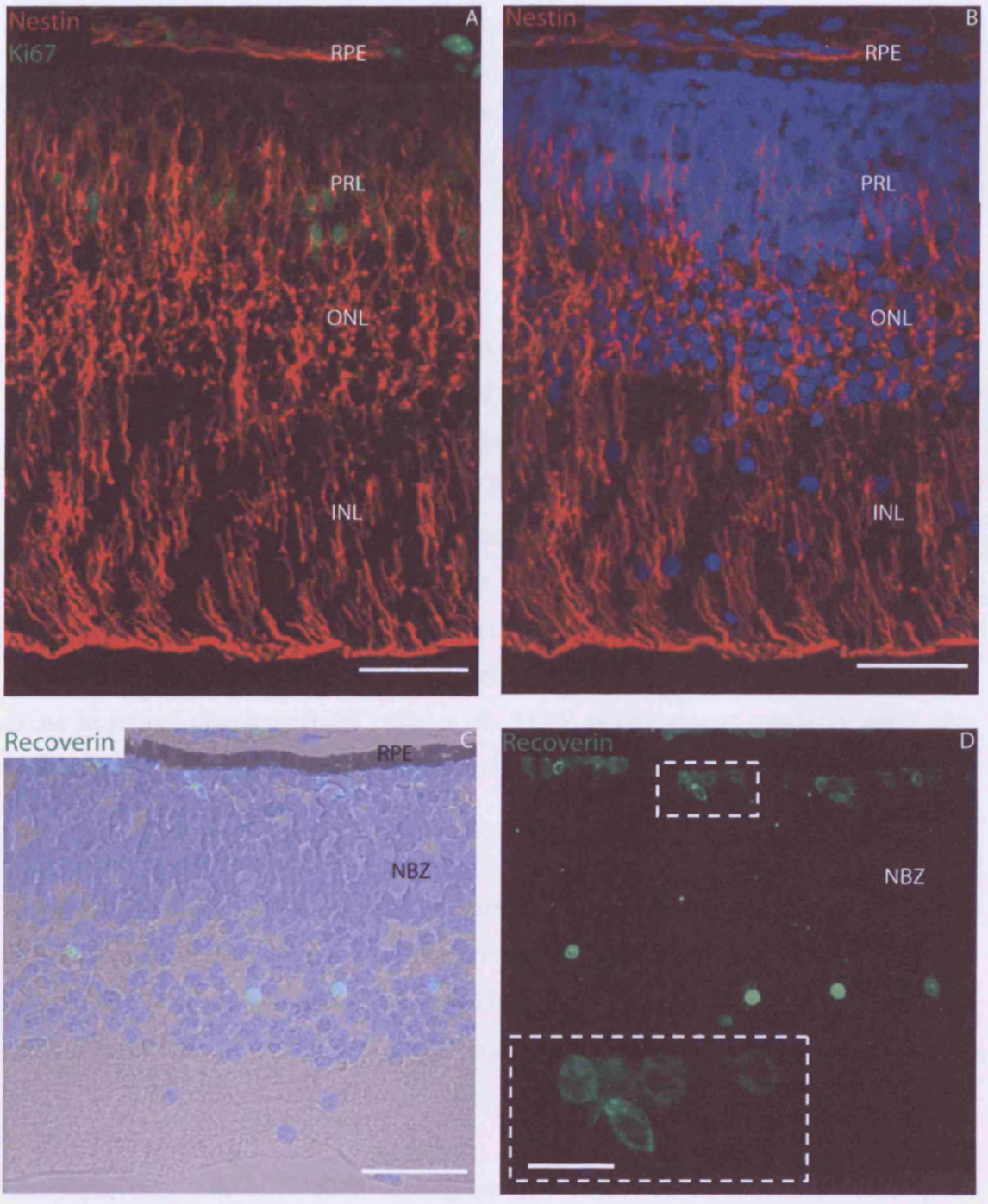

Figure 3.1 Human foetal retina at 12 weeks gestation
Human foetal retina was sectioned and stained for several markers to identify the expression profile of cells within the developing retina, with particular regard to some of the antigens used for immunocytochemistry on the human foetal retinal progenitor cells obtained from ReNeuron. (A) Section through a 12 week human retina stained for nestin and Ki67. (B) Human retina stained for nestin and DAPI to reveal the nuclei. (C, D) At this time point cells adjacent to the RPE (retinal pigment layer) and the INL (inner nuclear layer) are positive for recoverin. PRL: photoreceptor layer; ONL: outer nuclear layer; NBZ: Neuroblastic zone. Scale bar = 50 μm and 20 μm in the boxed region of image D. (Courtesy of Dr. A. Vugler).

3.1.1.2 Neurofilament markers

Almost all neural cells contain neurofilaments (NF) composed of three subunit polypeptides, namely the 70kD, 160kD and 200kD neurofilament proteins, of which the latter two are specifically stained for in this work. In the human retina the expression of these neurofilaments is present in the axons of the retinal ganglion cells (Kivelä *et al.*, 1985). As the retina matures NF200 is up-regulated compared to the other two triplet proteins.

3.1.1.3 Short wavelength cone opsin

The short wavelength cone opsin (S-opsin) is one of the specific photopigments for cone photoreceptors and is first detected in the human foetal retina (around the developing fovea) from week 11, with expression gradually spreading throughout the entire retina by week 20 (Cornish *et al.*, 2004). Short-wavelength sensitive cones (S cones) have been detected in foetal, postnatal and adult human retinae (Xiao and Hendrickson, 2000) and are responsible for blue colour vision (Dacey, 1999). In many mammals, cones constitute less than 5% of the entire population of photoreceptors (Carter-Dawson and LaVail, 1979), and because of the sequential order of 'birth' that exists in retinal development (Cepko *et al.*, 1996) cones appear relatively early after appearance of the first cell type (ganglion cells).

3.1.1.4 Rhodopsin kinase

Rhodopsin kinase is a rod and cone photoreceptor specific enzyme that contributes to the termination of the phototransduction pathway by phosphorylating the photoactivated opsin photopigments (Young *et al.*, 2003). Mutations in the rhodopsin kinase gene cause Oguchi disease, a form of night blindness (Sokal *et al.*, 2002).

3.1.1.5 Recoverin

Recoverin is a 23kDa calcium-binding protein expressed in retinal photoreceptors (Wiechmann, 1996) that regulates the phototransduction cascade by inhibiting rhodopsin kinase at high calcium levels, thus preventing the phosphorylation of photoexcited rhodopsin (Ames *et al.*, 2006). Recoverin immunoreactivity has been localised to photoreceptor cells, a subpopulation of cone bipolar cells, and a sparse population in the ganglion cell layer (Wiechmann, 1996; Yan and Wiechmann, 1997). In humans, by week 13 in gestation, recoverin expression is clearly evident (figure 3.1) and gradually reduces until birth (Yan and Wiechmann, 1997).

3.1.1.6 ß III tubulin

ß III tubulin is a neuron-specific microtubule protein that is a marker for immature neurons and ganglion cells. The expression of ß III tubulin coincides with the onset of differentiation (Sharma and Ehinger, 1997). Recent evidence suggests that certain horizontal, amacrine and cone photoreceptors also stain positively for this protein. These cells all belong to the early phase of cell birth in the neural retina (Sharma and Netland, 2007).

3.1.1.7 Rhodopsin

Rhodopsin is the photopigment that mediates phototransduction in all rod photoreceptors. It is a G-protein-coupled receptor that is chemically linked to the chromophore 11-cis-retinal (Maeda *et al.*, 2003; Palczewski and Saari, 1997). Rhodopsin is the most reliable marker of rod photoreceptors, however its synthesis only occurs 30 days after the birthdate of the photoreceptor itself (Cepko, 1996). This suggests that the expression of rhodopsin is determined by the position it has in the retina and not the birthdate, implying that neighbouring cells or local signals influence the expression of rhodopsin.

3.1.1.8 Ki67

Gerdes *et al.*, (1983) identified a human nuclear protein Ki67 that was expressed within the nuclei of all human cells in all active parts of the cell cycle (G1, S, G2 and mitosis) but was absent in non-dividing cells (G0). As Ki67 is confined to the nucleus and is present during the cell cycle it suggests an essential role in the regulation of cell cycle division, this has been confirmed by the introduction of Ki67 antigen-specific antisense oligonucleotides that results in the inhibition of proliferation in the cell line targeted (Schülter *et al.*, 1993).

3.1.1.9 Nrl

The Neural Retina Leucine zipper (NRL) transcription factor is essential for the development and differentiation of rod photoreceptors through its role as an activator of various target genes. Nrl $^{-/-}$ null mutant mice lack any sort of rod photoreceptor function and rhodopsin immunoreactivity (Mears *et al.*, 2001), demonstrating the importance of NRL in functional rod photoreceptor formation.

3.1.1.10 Crx

The Crx (Cone rod homeobox) gene encodes a 299 amino acid protein. In the adult, Crx is expressed in photoreceptors and pinealocytes. In E12.5 mice, Crx expression is detectable within the retina, where its main function is to bind to promoter regions of rhodopsin and several other photoreceptor-specific genes in order to activate these genes. Crx therefore plays an important role in retinal development. There is also evidence that Crx and Nrl act synergistically to activate rhodopsin expression (Chen *et al.*, 1997).

3.1.1.11 Sox2

The SRY box (SOX) proteins comprise a family of transcription factors that contain DNA-binding High Mobility Group (HMG) domains that are generally expressed during early cell fate decisions (Pevny and Lovell-Badge, 1997). Sox1, Sox2 and Sox3 are initially expressed in the anterior neural plate and invaginating optic vesicle. During the formation of the optic cup, Sox1 and Sox3 are down-regulated, while Sox2 is maintained and restricted to neural retina cells (Kamachi *et al.*, 1998). However, as neural precursors differentiate, the expression of Sox2 is down-regulated (Pevny and Lovell-Badge, 1997). To date, the importance of Sox2 in the nervous system has been highlighted by mis-expression studies in mouse, chick and Xenopus that suggest the role of Sox2 is to maintain neural progenitor identity.

3.1.1.12 Chx10

Chx10, a homeobox gene expressed in the presumptive neural retina is essential for eye development as mutation in the gene results in microphthalmia. Chx10 regulates the proliferation of retinal progenitor cells, rather than influencing the differentiation of the neural retinal cells (Burmeister *et al.*, 1996). Chx10 also specifies the neural retinal region and inhibits other transcription factors in order to specify this region.

3.1.1.13 Hes1

Hes1 is a member of the basic helix-loop-helix (bHLH) transcription factor family and acts as a key regulator of the proliferation process in retinal progenitor cells. In Hes1 mutant mice embryos, cell proliferation in the retina is severely impaired (Ohsawa and Kageyama, 2008).

3.2 Experimental design and objectives

The purpose of this set of experiments was to characterise the GuRt09 and GuRt05 clonally immortalised cell lines, and to elucidate their potential to exhibit progenitor cell proteins and properties. In order to fully characterise the cell lines a comparative study was run in parallel whereby primary cells were taken from 10-13 week old neural retina without undergoing immortalisation. Due to the finite nature and restricted number of the unimmortalised cells, there was a limitation on the range of experiments that could be undertaken. All cells were cultured at a density of 10,000 cells /cm^2 on laminin-coated dishes (for the immortalised cell lines) and fibronectin-coated dishes (for the unimmortalised cells) for a 7 day period prior to immunocytochemical analysis or total RNA extraction using Trizol reagent according to manufacturer's instructions. Specificity of immunostaining was tested by omission of the primary antibody, and absence of positive staining indicated specificity of the secondary antibody. Where possible, pre-incubation of primary antibody with immunising peptide was also undertaken. A positive control sample of human retinal cDNA from a library was used as a template for the primers used in the RT-PCR reactions to further confirm the primers' specificity.

The first aim of this thesis was to examine the characteristics of the cell lines supplied to us by ReNeuron. This was performed by examining the immortalised cells at the permissive and non-permissive temperatures, to determine whether there were any visible differences when the cells were permitted to exit the cell cycle at the non-permissive temperature of 37°C.

3.2.1 Substrate considerations

Throughout the thesis it was decided that the immortalised cells would be grown on laminin-coated flasks and dishes. The rationale for this was the report that plating retinal progenitor cells on laminin matrices causes an increase in rod photoreceptor differentiation (Levine *et al.*, 2000).

3.3 Results

In this study, the immortalised human foetal retinal progenitor cell lines, GuRt09 and GuRt05, were characterised individually and in comparison with an unimmortalised retinal progenitor cell culture, GS076. All cell types were analysed morphologically (figure 3.2) and appeared to exhibit differences. While the GuRt09 and GuRt05 cell lines tended to be spindle-like, the GS076 cells had a larger cytoplasm and were more rounded in morphology. This could be a consequence of the immortalisation procedure of the GuRt09 and GuRt05 cell lines. In this study the GuRt09 and GuRt05 cell lines were passaged long term (up to 35 passages), whereas it was very difficult to culture the primary unimmortalised cells beyond 10 passages, even when they were maintained on fibronectin-coated flasks, and cultured in serum-defined medium. In addition, selection pressure under culture conditions would probably lead to less heterogeneity within the GS076 cells, making it more difficult to compare early and late passage cultures.

In immunocytochemical analysis, GuRt09 cells, cultured at 33°C expressed the intermediate neurofilament protein nestin, a marker of neural progenitor cells (figure 3.3 and 3.4). This expression was also apparent at 37°C (figure 3.5), the non-permissive temperature, at which cells should exit the cell cycle and potentially differentiate. At the permissive temperature, conducive with proliferation, the cells expressed Ki67, NF160, NF200, recoverin and rhodopsin kinase. The expression of rhodopsin, Crx and Nrl was not detected at 33°C. Interestingly, the cells did not express Sox2, the transcription factor expressed early in the developing neural retina. However as neural precursors differentiate, the expression of Sox2 is down-regulated (Pevny and Lovell-Badge, 1997) consistent with the notion that these cells used here may be beginning to differentiate. To further characterise the cells, they were cultured at 37°C to determine whether inactivation of the immortalising oncogene would be sufficient to permit signs of differentiation or morphological differences. The results showed that markers of a more differentiated state such as ß III Tubulin (figure 3.5), an early neuronal and ganglion cell marker, could not be detected, nor was the expression of rhodopsin, S-opsin, Crx or Nrl detectable. The expression patterns of nestin, NF160, NF200, rhodopsin kinase and Ki67 were apparently no different from these observed in GuRt09 cells cultured at 33°C. The detection of Ki67, a proliferation marker, was unexpected at 37°C because at this temperature the SV40 oncogene should be conditionally switched off, therefore allowing cells to exit the cell cycle.

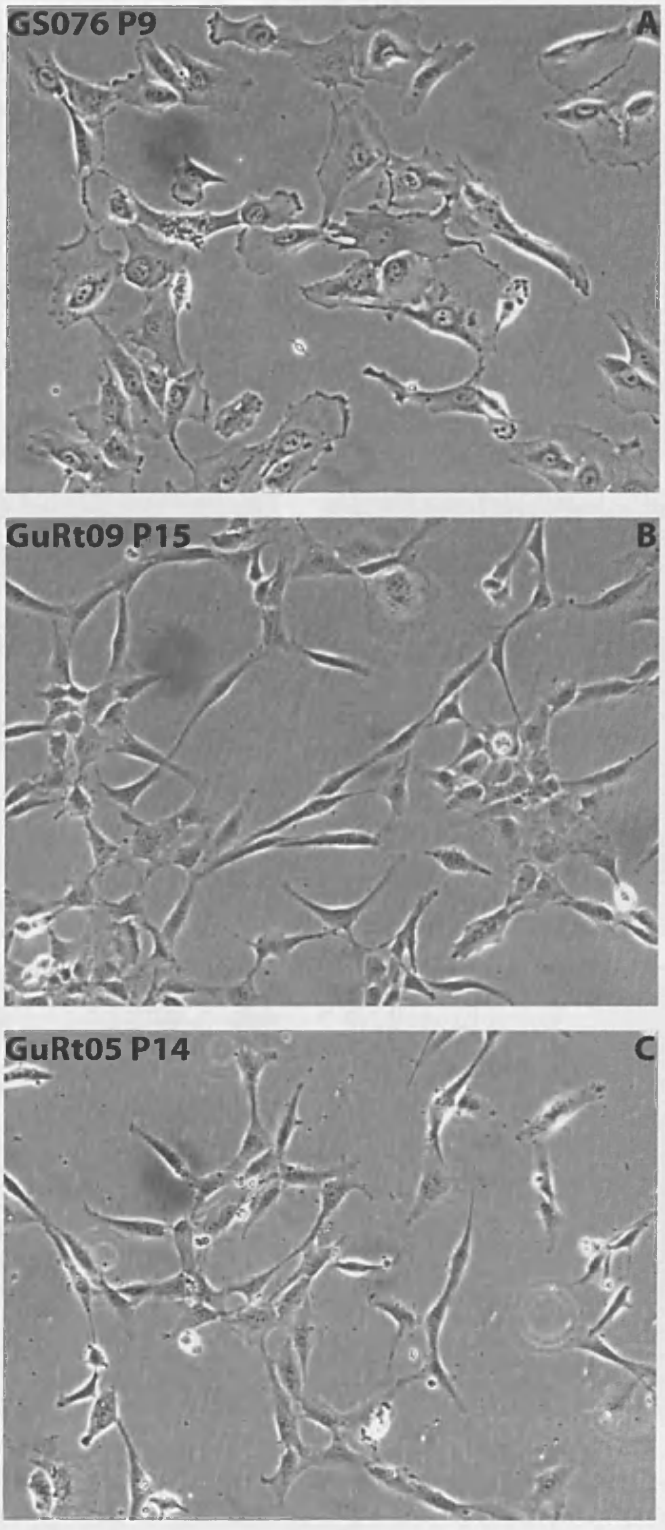

Figure 3.2 Primary and immortalised human foetal retinal progenitor cells
Phase contrast images of progenitor cells taken from the neural retina of 10-13 week old aborted foetuses. (A) GS076 (P9) cells are primary cultures, whilst (B,C) are cells that have undergone clonal immortalisation to generate GuRt09 and GuRt05 cell lines. The immortalised cells appear more spindle-like whilst the GS076 (A) appear more flattened with larger cytoplasms.

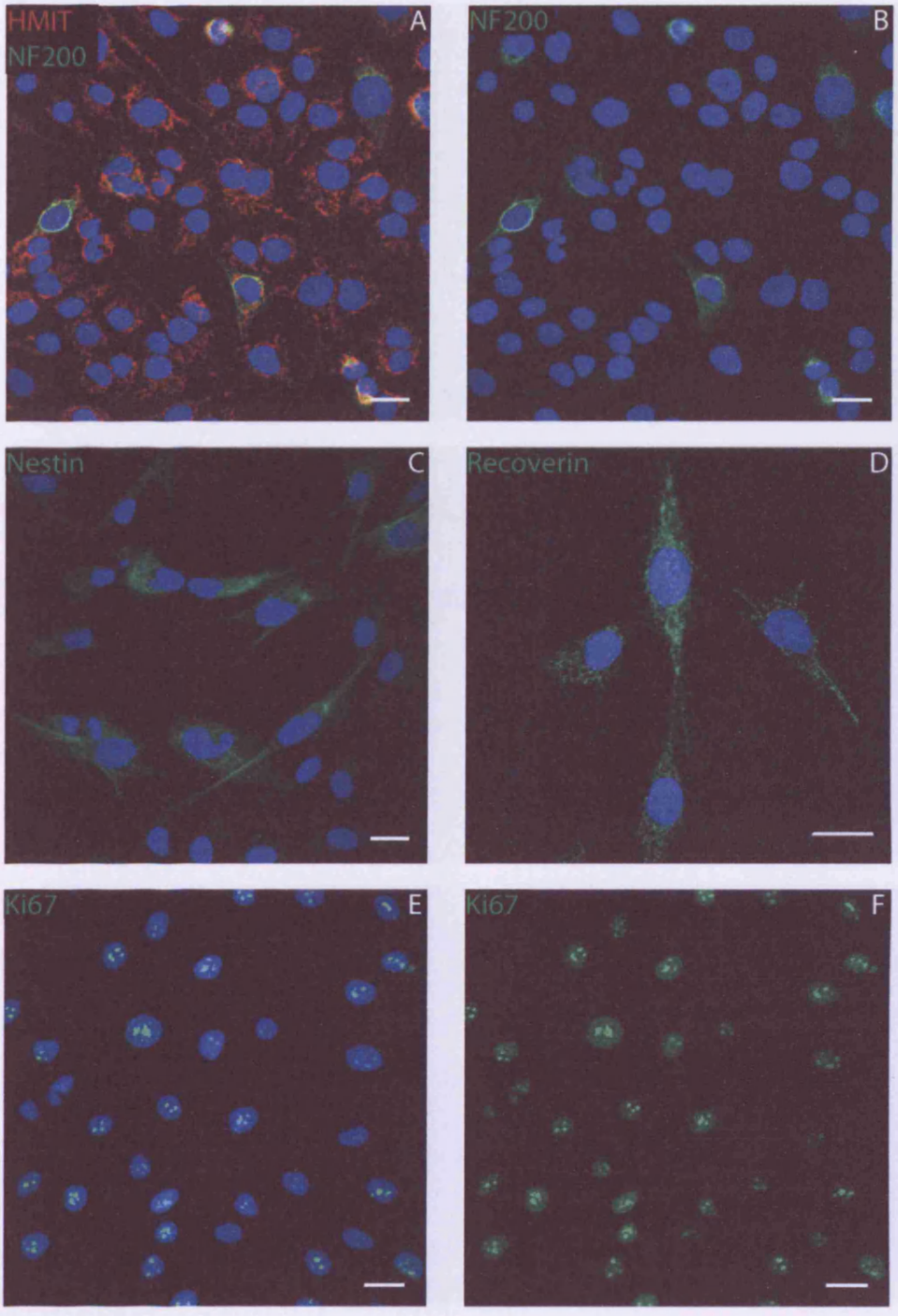

Figure 3.3 Expression profile of GuRt09 at the permissive temperature
Confocal images of GuRt09 (P15-P20) cells grown on laminin at 33°C in human medium for 7 days. (A) Cells stained for human mitochondrial marker (HMIT) and NF200. (B) Expression of NF200 alone. (C) Expression of the neuroectodermal marker nestin. (D) Recoverin expression. (E, F) Cells are positive for the proliferation antigen of Ki67. Nuclei were counterstained with DAPI and are blue in all images. Scale bar = 20 µm.

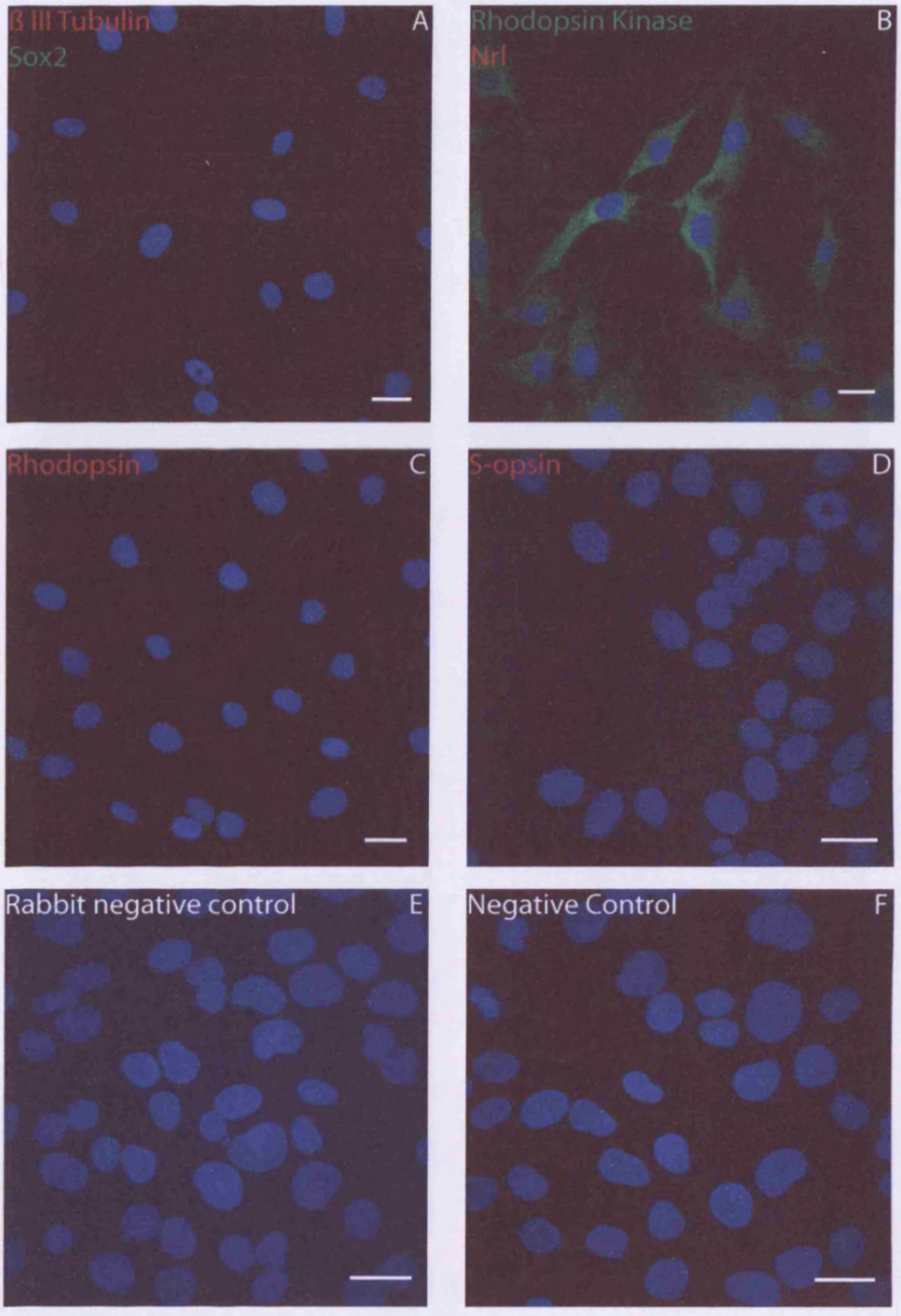

Figure 3.4 Expression profile of GuRt09 at the permissive temperature
Confocal images of GuRt09 (P15-P20) cells grown on laminin at 33°C in human medium for 7 days. (A) ß III Tubulin and Sox2 were undetectable in the GuRt09 cell line. (B) Cells were positive for the rhodopsin kinase antigen, however Nrl was undetected via immunocytochemistry. (C) Rhodopsin expression was not detectable at this stage, neither was S-opsin (D). (E,F) Run in parallel with all staining samples were negative controls where the primary antibody was omitted from the protocol to detect any non-specific staining.
Scale bar = 20 µm.

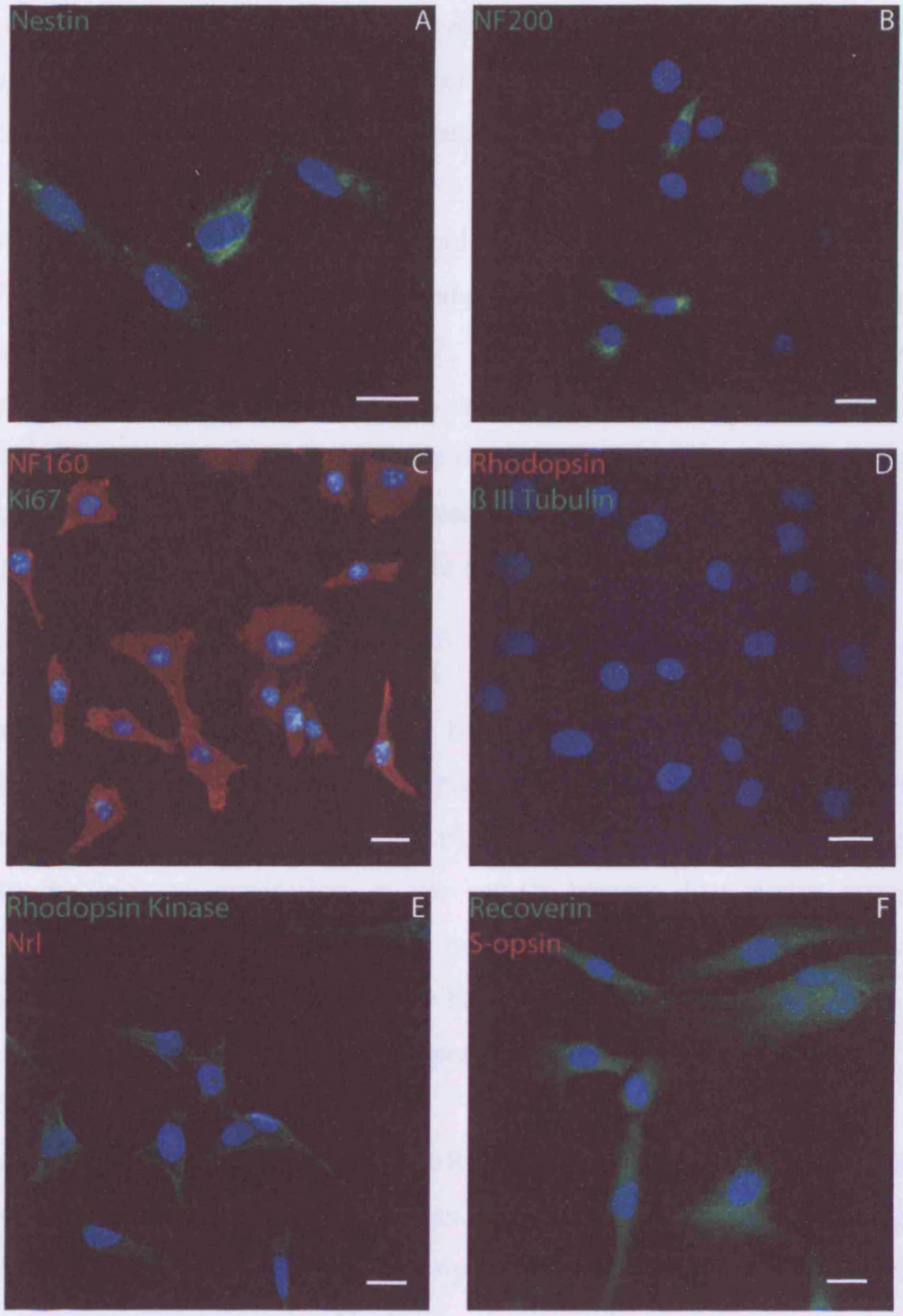

Figure 3.5 Expression profile of GuRt09 at the non-permissive temperature
Confocal images of GuRt09 (P18-21) cells grown on laminin at 37˚C in human medium for 7 days. (A) Cells stained for nestin. (B) Expression of NF200 alone. (C) Expression of NF160 and Ki67. (D) Rhodopsin and ß III Tubulin were undetectable. (E) Cells are positive for rhodopsin kinase, but Nrl was not detected. (F) Cells were positive for recoverin and S-opsin was not detected. Nuclei were counterstained with DAPI and are blue in all images. Scale bar = 20 µm.

Similar expression patterns were observed for the GuRt05 clonal cell line, at both permissive and non-permissive temperatures the cells were immunoreactive for the same sets of markers (figures 3.6 and 3.7) as the GuRt09 cell line. Again there was no detection of ß III tubulin, the neural progenitor transcription factor Sox2, S-opsin, or later proteins involved in rhodopsin expression, Crx and Nrl, or rhodopsin itself.

The GS076 primary unimmortalised cells showed some similarities with the immortalised cell lines as they expressed nestin, NF160 and NF200 (figure 3.8). Interestingly, not all cells expressed Ki67, indicating that a proportion of cells were either senescent or post-mitotic. This was in contrast to the GuRt09 and GuRt05 cell lines where all cells were positive for this marker. In comparison to the immortalised cell lines, the primary cells were immunoreactive for a subset of later neuronal markers including ß III tubulin and S-opsin, however rhodopsin expression was not detected.

Further analysis of the genes expressed by the cell lines and primary cells was determined by RT-PCR. All primers were first tested to amplify cDNA from a human retinal library as a positive control, and indeed were able to amplify the correct size amplicon from the retinal library (figure 3.9), with the exception of nestin, which could be due to the lack of expression in the retinal library. The nestin primers were nevertheless used, and as the correct size amplicon was amplified from immortalised and unimmortalised cell types without any band appearing in the no RT or no template controls, these primers were judged to be specific.

Interestingly, all the cells expressed the mRNAs for S-opsin and ß III tubulin (figure 3.10), despite the fact that the gene products were not detected at the protein level. The cells were also positive for other transcription factors that are important to neural progenitor maintenance, including Hes1. There was no detection of rhodopsin, Crx, Nrl at the transcriptional level. Overall, there were no apparent differences in the expression patterns of genes examined in the GS076 cells and the immortalised cell lines, and when the cell lines were cultured at 33°C and 37°C the RT-PCR analysis similarily showed no apparent differences.

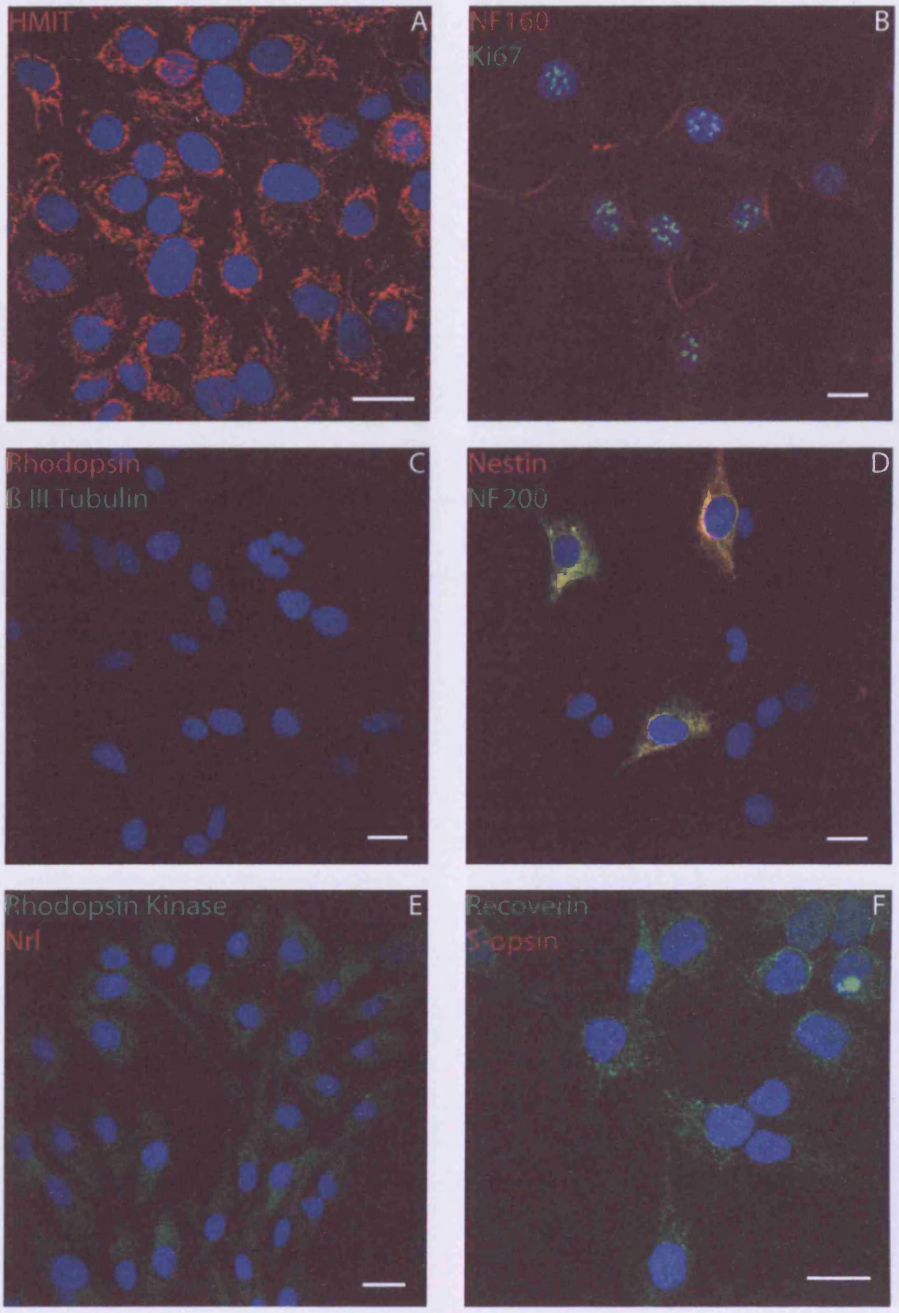

Figure 3.6 Expression profile of GuRt05 at the permissive temperature
Confocal images of GuRt05 (P17-20) cells grown on laminin at 33°C in human medium for 7 days. (A) Cells stained for Human Mitochondrial Marker (HMIT). (B) Cells expressing NF160 and Ki67. (C) Rhodopsin and ß III Tubulin were undetectable. (D) Cells were positive for nestin and NF200 and the majority of cells co-expressed both neurofilament proteins. (E) Cells were positive for rhodopsin kinase, but Nrl was not detected. (F) Cells were positive for recoverin and S-opsin was not detected. Nuclei were counterstained with DAPI and are blue in all images. Scale bar = 20 µm.

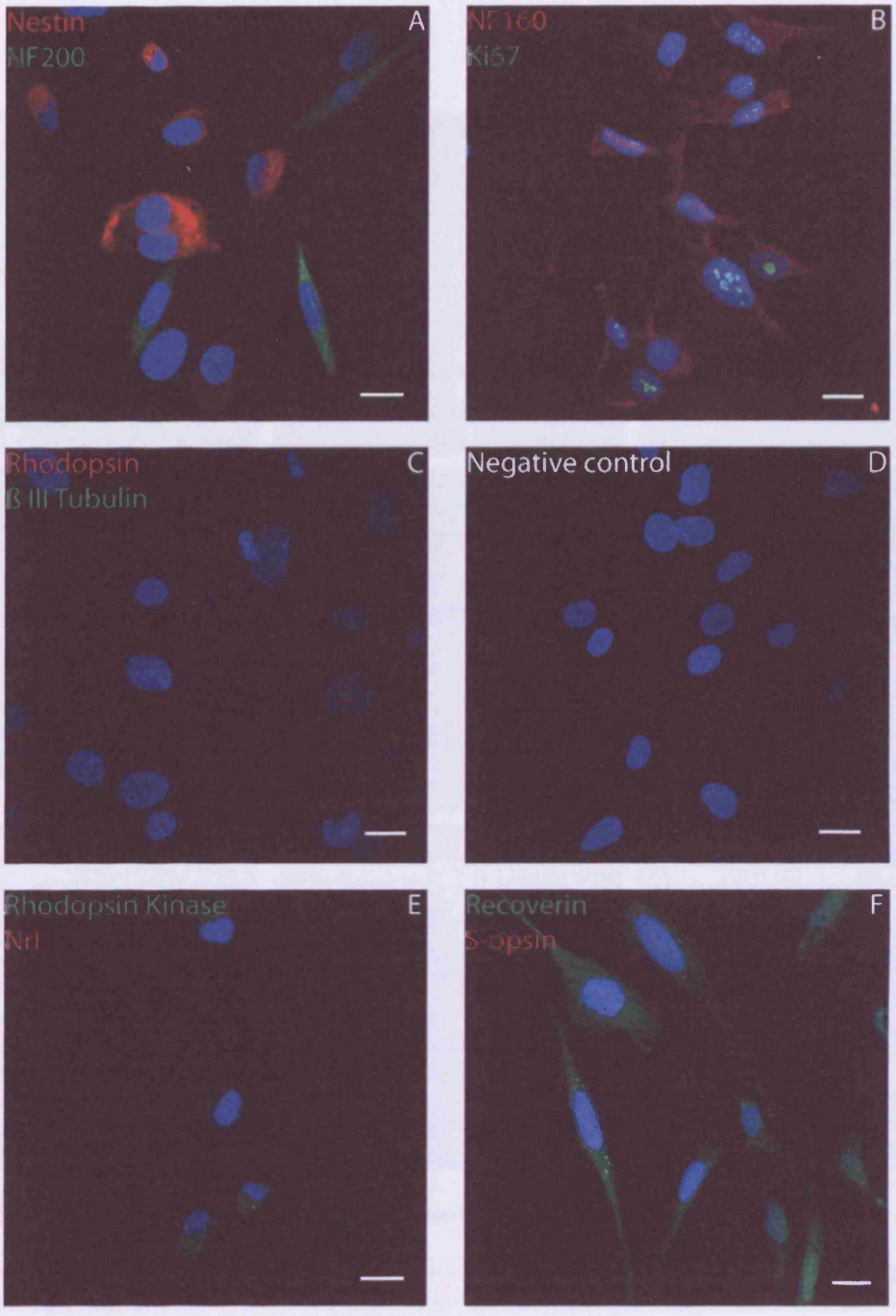

Figure 3.7 Expression profile of GuRt05 at the non-permissive temperature
Confocal images of GuRt05 (P15-20) cells grown on laminin at 37°C in human medium for 7 days. (A) Cells were positive for nestin and NF200. (B) Cells expressed NF160 and Ki67. (C) Rhodopsin and ß III Tubulin were not detected. (D) Negative controls were run in parallel with all samples to ensure no non-specific staining. (E) Cells were positive for rhodopsin kinase, but Nrl was not detected. (F) Cells were positive for recoverin and S-opsin was not detected. Nuclei were counterstained with DAPI and are blue in all images. Scale bar = 20 µm.

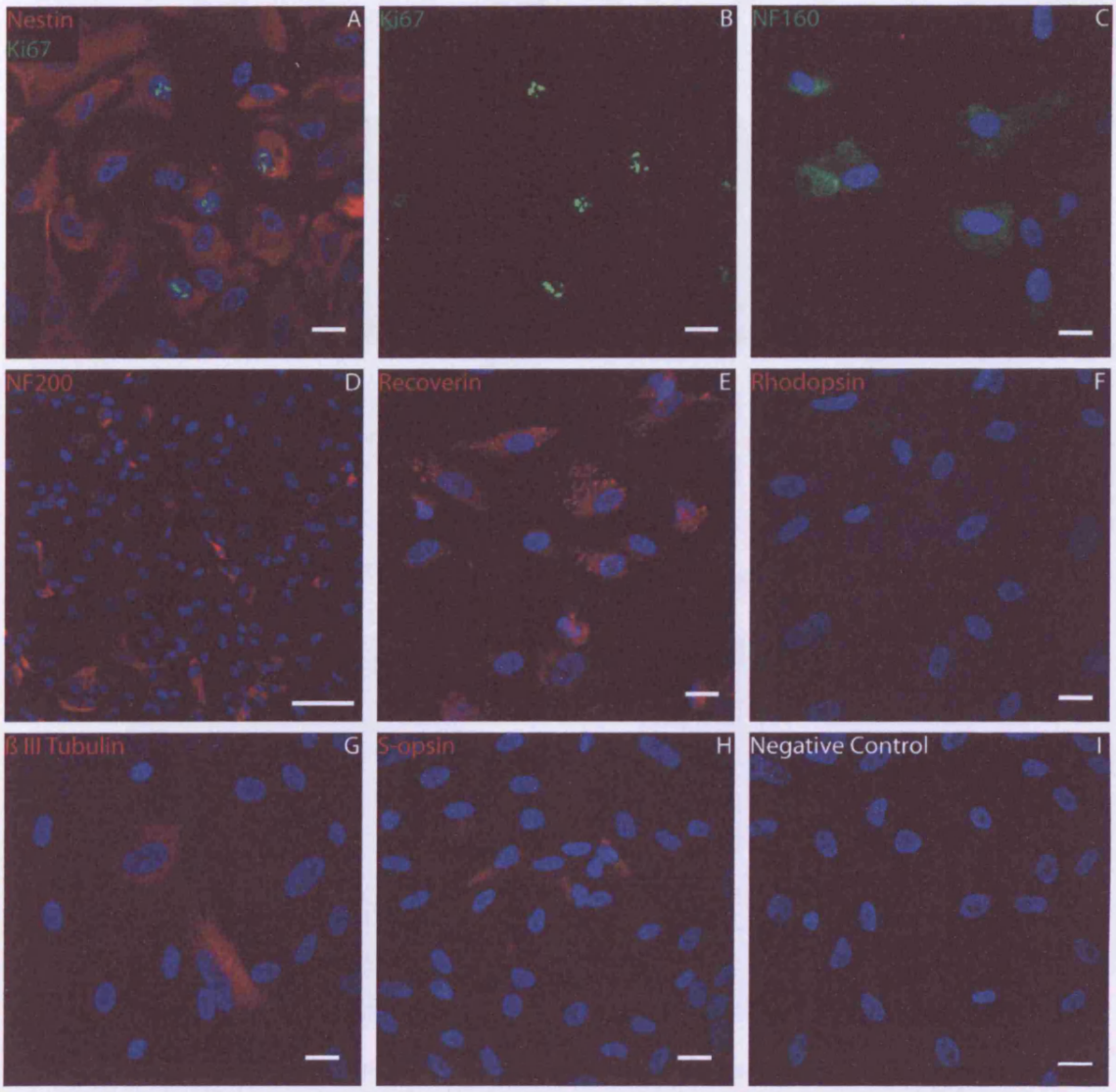

Figure 3.8 Expression patterns of retinal markers in GS076, unimmortalised human foetal retinal progenitor cells

Cells were obtained from 10-13 week old foetuses and cultured on fibronectin-coated dishes in 5% FBS supplemented human medium at 37°C. These confocal images were taken of GS076 (P10) cells. (A) Cells were positive for nestin and a portion of these cells were positive for ki67 antigen (B), however not all cells were actively proliferating. Cells were also positive for (C) NF160, (D) NF200, (E) Recoverin, (G) ß III Tubulin and (H) S-opsin. (I) Negative control run in parallel with this sample of immunocytochemistry, the absence of positive staining demonstrates the specificity of the secondary antibodies used. (F) Rhodopsin was not detected by immunocytochemistry. Scale bar = 20 µm.

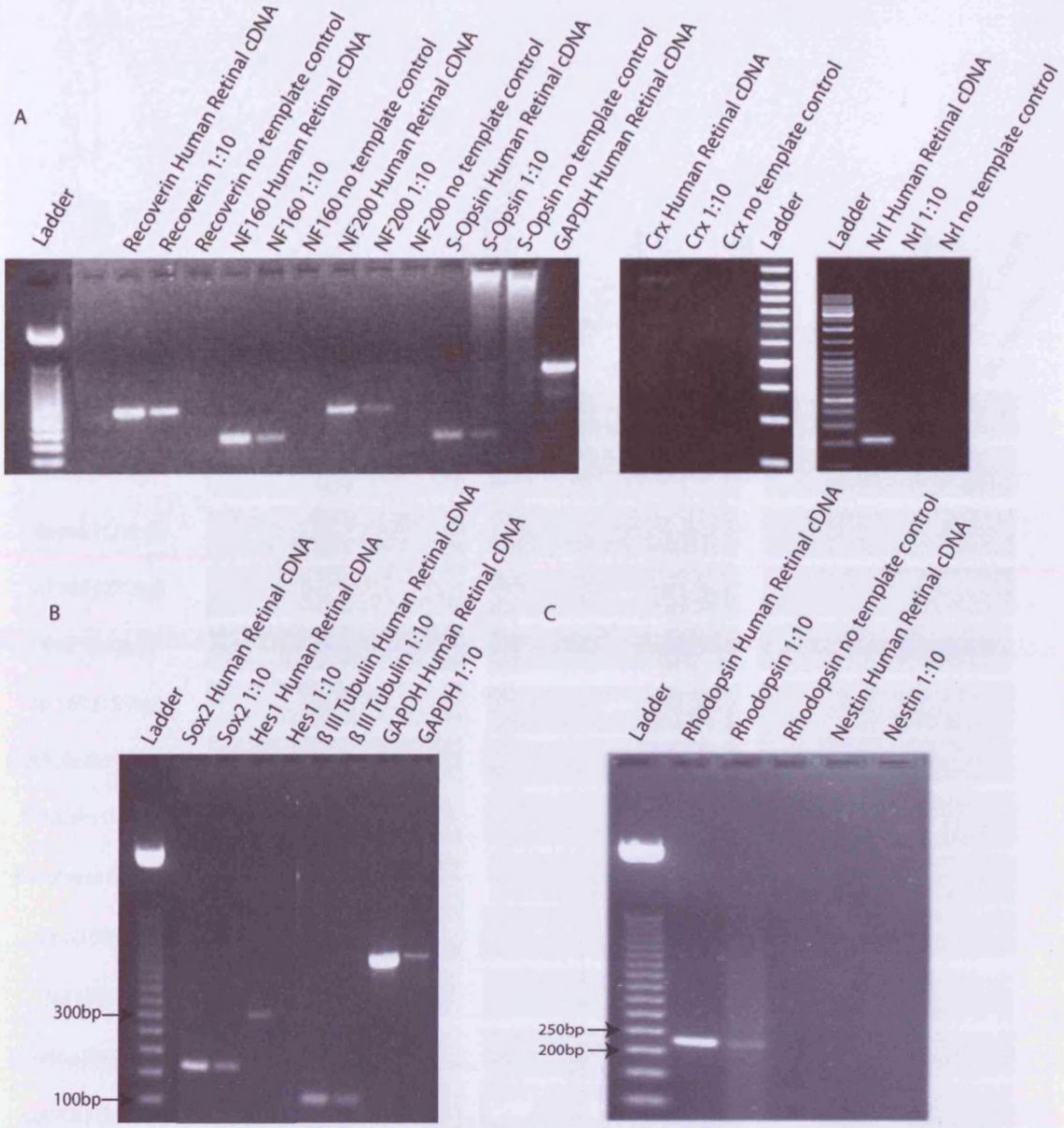

Figure 3.9 PCR analysis of discriminatory markers in human retinal cDNA library
The cDNA from a human retinal library was used to screen the primers prior to RT-PCR analysis of the GuRt09, GuRt05 cell lines and primary GS076 primary cells. The retinal cDNA was used as a positive control to ensure that the primers were able to amplify the correct sized amplicons. All PCR reactions performed using the retinal cDNA were run using stock solutions of cDNA, a 1:10 dilution of the stock cDNA and a no template control (only shown in A). (A) The specific markers amplified were recoverin (282bp), NF160 (155bp), NF200 (271bp), S-Opsin (148bp), a positive GAPDH control (550bp), Crx (1059bp) and Nrl (212bp). (B) Amplicons for Sox2 (170bp), Hes1 (307bp) and ß III Tubulin (115bp) were also detected. (C) Rhodopsin (227bp) was amplified from the retinal cDNA, however nestin (176bp) was not detected with this cDNA sample.

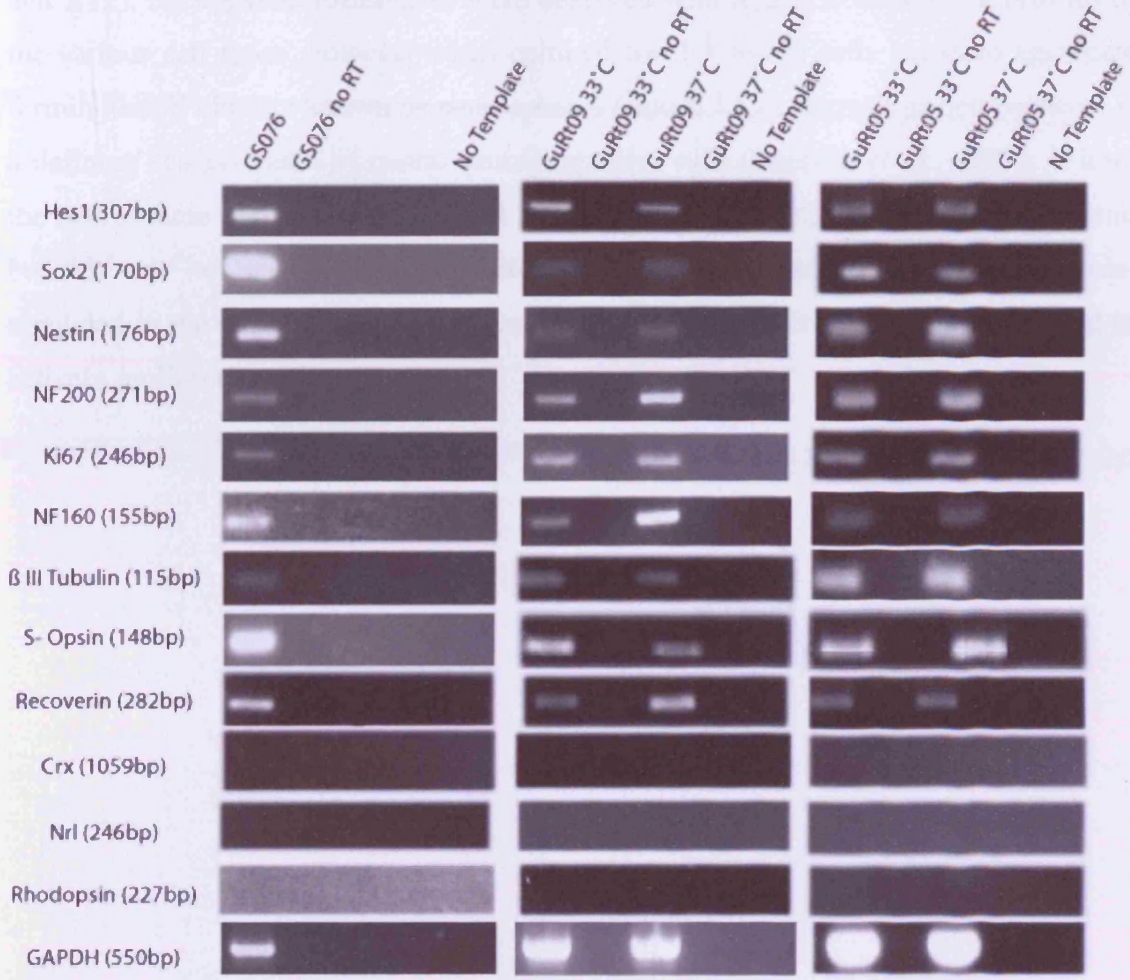

Figure 3.10 RT-PCR analysis in GS076 primary cells and GuRt09 and GuRt05 cell lines
RNA was extracted from cells cultured in medium for 7 days and cDNA was used to amplify particular genes or markers. GAPDH was used as a positive control throughout. As an internal control a sample of RNA that had not been reverse transcribed was used as a no RT control in order to distinguish genomic contamination. GS076 cDNA was run in parallel with GuRt09 cDNA cultured at 37°C. Crx, Nrl and Rhodopsin were not detected via RT-PCR.

Immortalised and unimmortalised cells were routinely cultured on laminin or fibronectin coated dishes respectively. This was due to the finding reported elsewhere that plating retinal progenitor cells on laminin matrices causes an increase in rod photoreceptor differentiation (Levine *et al.*, 2000). However, cells were also cultured on plastic to ascertain any differences in expression patterns or morphology (figures 3.11 and 3.12). No apparent differences were observed with regard to expression profiles of the various cell types, however when cultured for 2-3 weeks cells began to aggregate forming small clusters known as neurospheres (figure 3.13). Growth as neurospheres is a defining characteristic of neural stem/progenitor cells (Sheedlo *et al.*, 2007). Within the neurosphere both the immortalised and unimmortalised cell types expressed nestin, but this was not seen in all cells. Interestingly, the expression of Ki67 was down-regulated in the GS076 neurospheres as opposed to the GuRt09 cells that contrived to actively proliferate.

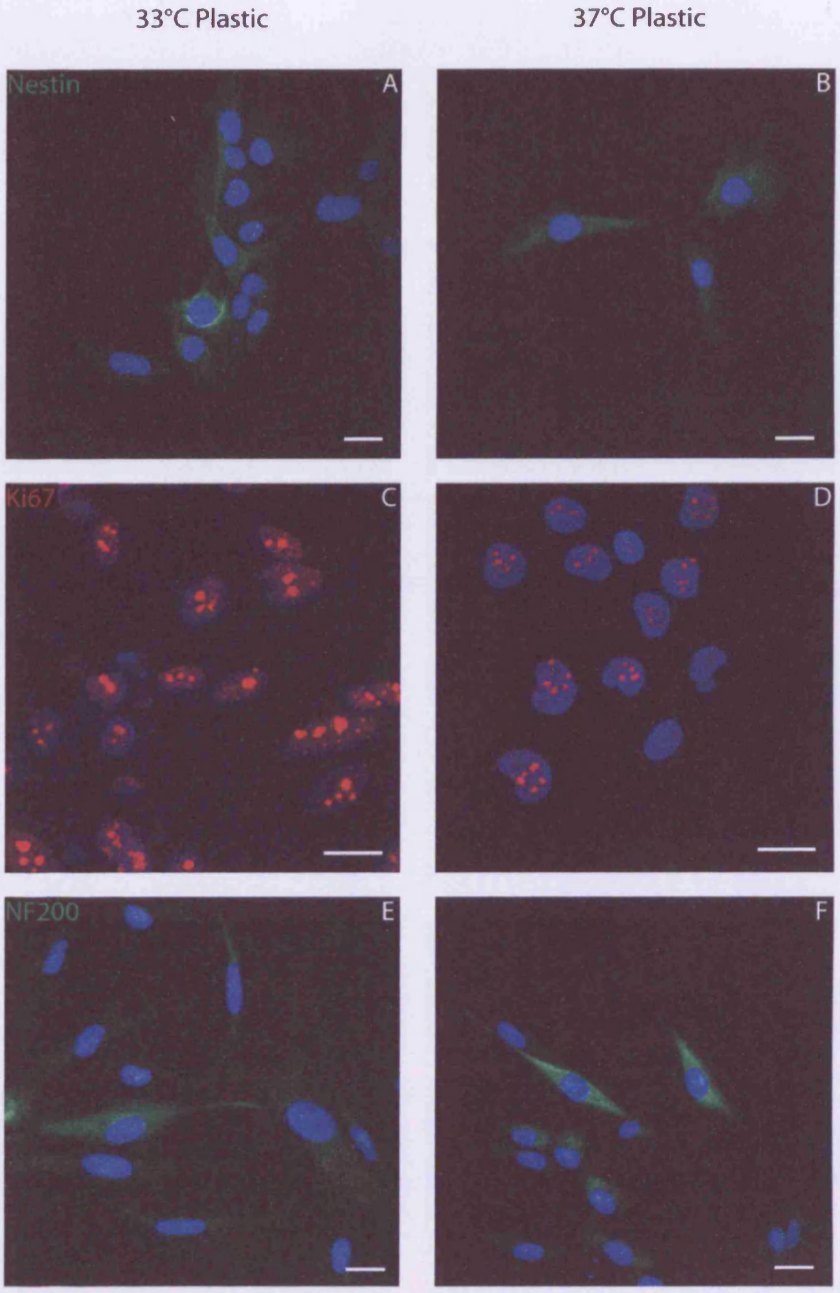

Figure 3.11 Expression profile of GuRt09 cell line on plastic
Confocal images of GuRt09 (P18-22) cells grown on plastic at 33˚C and 37°C in human medium for 7 days. (A, B) Cells stained for nestin showing similar levels of expression at both temperatures. (C, D) Cells were positive for the Ki67 antigen. (E,F) Cells were positive for NF200 expression. Nuclei were counterstained with DAPI and are blue in all images. Scale bar = 20 μm.

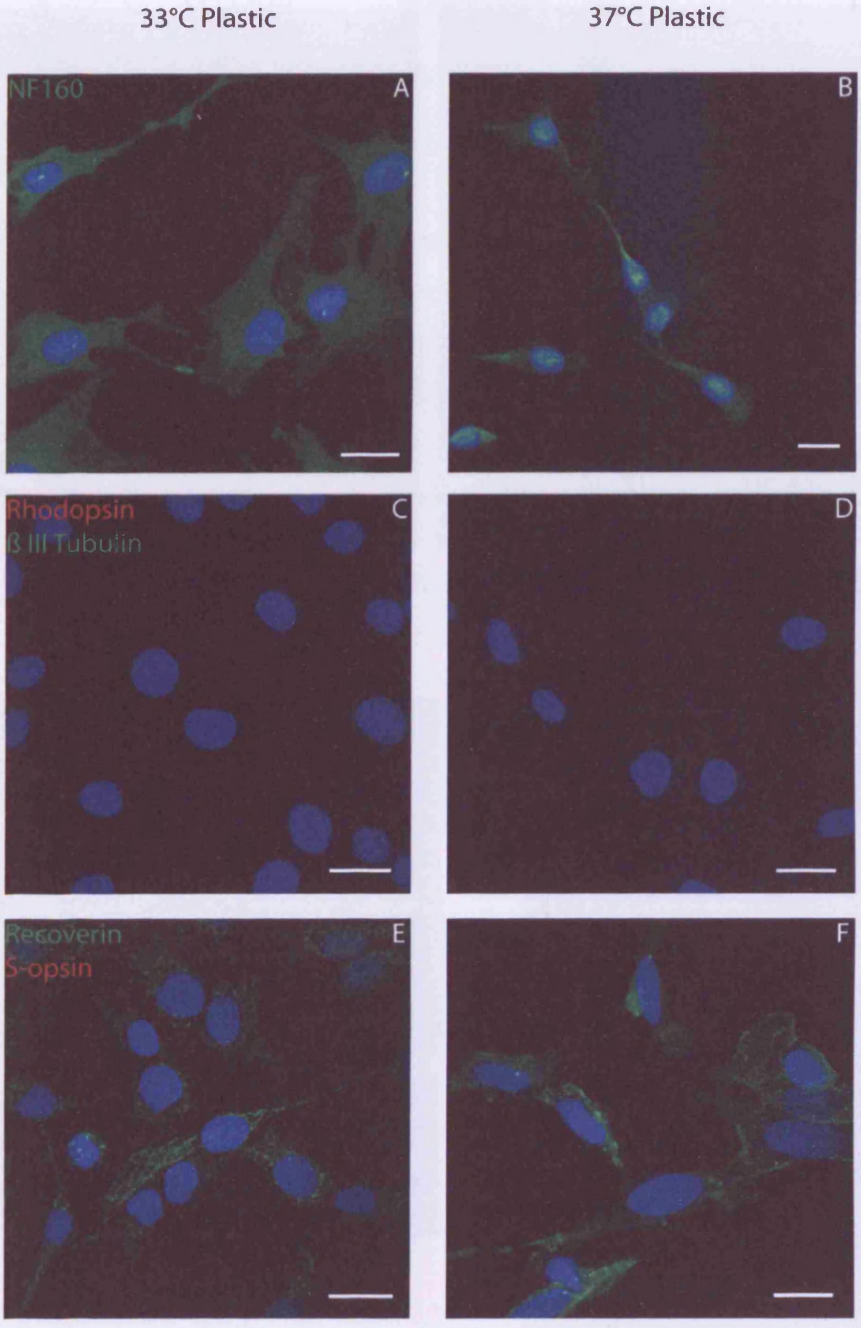

Figure 3.12 Expression profile of GuRt09 cell line on plastic
Confocal images of GuRt09 (P18-22) cells grown on plastic at 33°C and 37°C in human medium for 7 days. (A, B) Cells were positive for NF160 at both temperatures. (C,D) Rhodopsin and ß III tubulin were not detected at either condition. (E,F) Cells were positive for recoverin, however S-opsin was undetected via immunocytochemistry. Nuclei were counterstained with DAPI and are blue in all images. Scale bar = 20 µm.

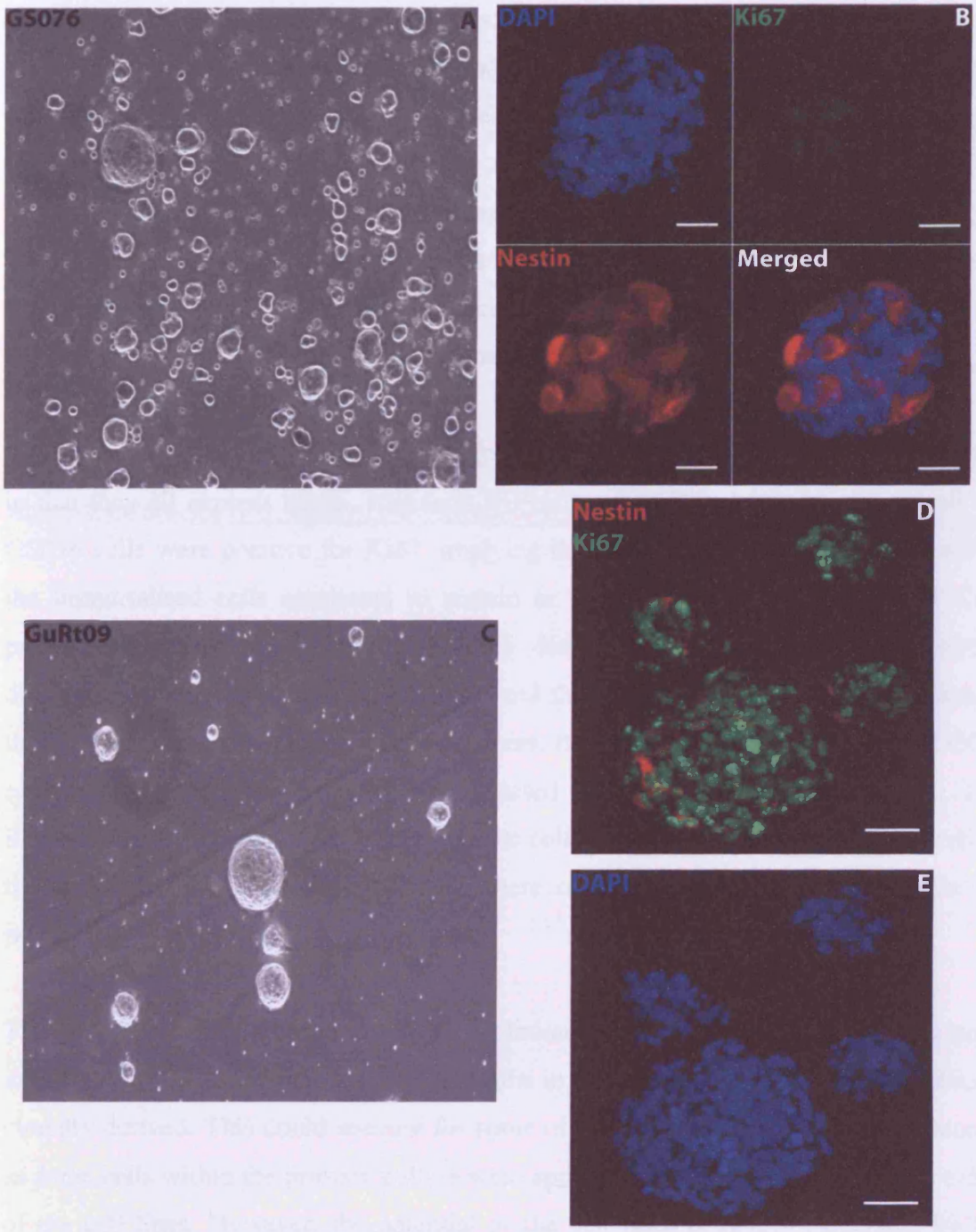

Figure 3.13 Formation of neurospheres by GuRt09 and GS076 cells
GuRt09 and GS076 cell cultures were seeded on to plastic dishes and left for a duration of 2-3 weeks, at which point the cells began to form neurospheres. (A) Phase-contrast image of the GS076 cell culture forming multiple neurospheres. (B) Confocal images of GS076 neurospheres staining positively for nestin and very weakly for Ki67. (C) The immortalised retinal progenitor cells also retained the ability to form spheres. (D,E) Confocal images of the GuRt09 spheres expressing nestin and Ki67. All nuclei were stained with DAPI. Scale bar = 20 μm.

3.4 Discussion

In summary, these studies have shown that the immortalised human foetal retinal progenitor cells express numerous retinal markers, including nestin, a protein widely used as a marker of neural progenitor cells. As cells begin to differentiate the expression of nestin is down-regulated (Lendahl *et al.*, 1990), therefore the continuous expression of nestin could be due to the fact that the cells have not been cultured in an environment that is conducive towards differentiation. Although one would expect down-regulation of nestin at 37°C, where the SV40 oncogene is switched off and therefore the cells should theoretically exit the cell cycle and begin to differentiate, without the appropriate cues perhaps the cells remain within the cell cycle, which would be consistent with the expression of Ki67 at the non-permissive temperature.

The primary GS076 cells are similar in expression patterns to the immortalised cell lines, in that they all express nestin, recoverin, NF160 and NF200. Interestingly, not all the GS076 cells were positive for Ki67, implying that many had stopped dividing, whilst the immortalised cells continued to remain in the cell cycle. The analysis of Ki67 protein expression is considered in more detail in subsequent chapters. Another difference between the immortalised cells and the primary cells was the expression at the protein level of the later neuronal markers, ß III tubulin and S-opsin in the GS076 cells, whilst these markers were not detected in the immortalised cell lines. This illustrates the possibility that even though the cells were taken from foetal neural retinal tissue at the same developmental stage, there could be significant differences in the potential of individual cells.

The GS076 cells are a heterogeneous population in which inevitable differences must exist in the potential of individual cells, whilst in comparison the immortalised cells are clonally derived. This could account for some of the differences in expression patterns as some cells within the primary culture were apparently more differentiated than either of the cell lines. However, the potential of the cell lines is clearly illustrated by the expression of S-opsin and ß III tubulin at the transcriptional level. Given that the cell lines express the mRNAs for these markers, it would be interesting to elucidate what molecular mechanisms and trophic factors might enable their translation and subsequent differentiation.

Chapter 4

The roles of fibroblast growth factor and epidermal growth factor on immortalised human foetal retinal progenitor cell lines

Chapter 4

The roles of fibroblast growth factor and epidermal growth factor on immortalised human foetal retinal progenitor cell lines

4.1 Introduction

There is a great deal of interest in the effects protein growth factors have on precursor cell behaviour, as they play a major role in determining cell fate (Giordano *et al.*, 2007). The presence of peptide growth factors have been shown to have mitogenic properties either by enhancing neuronal survival, neurite outgrowth or even neuronal differentiation in cell cultures *in vitro* (Kelley *et al.*, 1995). Due to these findings, importance has been placed on defining the role mitogenic growth factors exert on progenitor cell development. For instance a link between Fibroblast Growth Factor (FGF) and neural retinal differentiation in the developing embryo has been highlighted (Pittack *et al.*, 1997). This chapter will specifically concentrate on two such factors, the aforementioned FGF and Epidermal Growth Factor (EGF), in order to elucidate whether the removal or addition of these growth factors would influence the proliferation or differentiation of the immortalised retinal progenitor cell lines. Note that because of the potential of these factors and other mitogens to stimulate proliferation, the cells are normally grown in the presence of 10 ng/ml bFGF and 20 ng/ml EGF.

4.1.1 Basic fibroblast growth factor

Heparin binding growth factors (HBGF) make up a family of mitogens that have a high affinity for heparin (Hicks *et al.*, 1992a). Acidic and basic fibroblast growth factors (aFGF and bFGF) are the two main members of this family. Both factors are essential regulators of mitogenesis and differentiation of precursors cells in the CNS (Brickman, *et al.*, 1998). Basic Fibroblast Growth Factor (bFGF) is the most widely expressed of the two growth factors and is found at an earlier stage of development, E2 in the avian embryo (Guillemot and Cepko 1992; Mascarelli *et al.*, 1987).

4.1.1.1 Basic fibroblast growth factor and the neuroepithelium

During retinal histogenesis the neuroepithelium of the optic vesicles gives rise to two distinct tissues- the outer retinal pigment epithelium (RPE) and the inner neural retina layer. The development of both is controlled by the exact timing of specific interactions and signalling systems which include Bone Morphogenetic Protein (BMP) signalling responsible for specifying the RPE and fibroblast growth factors (FGFs) (see Figure 4.1)

that specify the neural retina (Dias da Silva *et al.*, 2007; Zuber and Harris, 2006). The role of FGF in neuronal histogenesis was further elucidated by observations in 22-24 stage chick embryos. Following retinectomy, beads were implanted into the eye with slow release bFGF that resulted in the transdifferentation of the retinal pigment epithelium (RPE) into the neural retina (Park and Hollenberg, 1989), even after it had begun to differentiate into RPE (Coulombre and Coulombre, 1965).

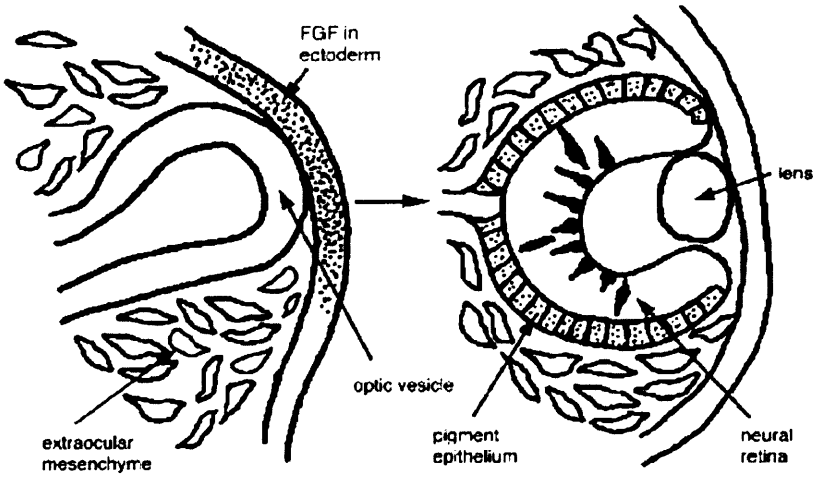

Figure 4.1 Model showing the anlage of the future neural retina and retinal pigment epithelium and the role bFGF plays in neural retinal induction
The optic vesicle is in close contact with the overlying ectoderm. A localised source of FGF from the ectoderm (stippled region) promotes neural retina development in this region. The presumptive retinal pigment epithelium (medial surface of the optic vesicle) does not receive this signal and therefore must rely on other signalling systems to promote differentation. (Adapted from Pittack *et al.*, 1997).

FGF has been detected in many organisms during embryonic development, including the chick eye (Mascarelli *et al.*, 1987) and the bovine and dogfish eyes (Lagente, 1986). *In situ* hybridisation studies led to the finding that cells within the developing rat retina (embryonic day 12 and postnatal day 1 embryos) express the mRNA for the FGF-receptor (FGF-R) (Wanaka, *et al.*, 1991). The use of neutralising antibodies to inhibit bFGF (also known as FGF2) in optic vesicles from embryonic day 1.5 chick resulted in the inhibition of neural differentiation in the presumptive neural retina but did not hinder the differentiation of the RPE (Pittack, *et al.*, 1997). These findings illustrate that FGF is not required for RPE differentiation from the optic vesicles and reinforces the importance of FGF in eye development.

4.1.1.2 Fibroblast growth factor and neural retinal development *in vitro*

Having established the importance of bFGF *in vivo*, studies were undertaken to observe the effects of bFGF on dissociated retinal cell types *in vitro*. In 1992, Guillemot and Cepko observed that dissected RPE explants, from 17-18 or 24-25 stage chick embryos cultured in the presence of aFGF and bFGF, lost their epithelial appearance and adopted a neuronal morphology after 3 to 4 days in culture. Immunocytochemical analysis confirmed the appearance of differentiation markers for retinal cell populations, including amacrine, horizontal and ganglion cell types. If differentiation of explanted RPE tissue was induced, then perhaps retinal precursor cells in the presence of exogenous FGF could respond in a similar manner?

The presence of bFGF mRNA in photoreceptors (Noji *et al.*, 1990) suggests that bFGF plays a role in photoreceptor development. This was investigated with the use of dissociated newborn rat retinal cells that were maintained as a monolayer in the presence of bFGF. These cells exhibited significant increases in rhodopsin expression compared to controls (Hicks and Courtois, 1992b). However, with increasing age of the cells, the effectiveness of bFGF in inducing rhodopsin expression diminished. This study found the effect of bFGF to be dose-dependent, with maximal effectiveness at 10 ng/ml. Other factors including EGF were without effect. The timing of induced differentiation is paramount, as is evident by the reduction in responsiveness to bFGF, as the cells get older (from newborn to 3 day postnatal). Therefore it is not only important to identify the most effective trophic factors, but also the juncture at which they should be administered.

4.1.2 Epidermal growth factor

In addition to being able to induce differentiation, FGF has also been shown to enhance the proliferation rate of neuronal precursor cells (Gensburger *et al.*, 1987). This is also an attribute shared by the other growth factor examined in this chapter, namely EGF. EGF is a potent mitogenic peptide, which stimulates cell growth and differentiation in a variety of cultured cells via specific EGF receptors (EGF-R) (Gray *et al.*, 1983; Werner *et al.*, 1988). In contrast to FGF, the activity of EGF is biased towards increased cell growth rather than differentiation. This point has been illustrated in dissociated neuronal progenitor cells from embryonic and neonatal rat retinae treated with 20 ng/ml EGF. The cells exhibit an increase in total cell numbers after the first 2 days in culture compared to control cells (Anchan *et al.*, 1991). Of particular relevance to work in this

thesis was the effect both bFGF and EGF had on human foetal retinal cells. In cultures with FGF or EGF alone the proliferative effects would diminish after a 2 month period, whilst in the long term the combined EGF and bFGF treated cultures continued to proliferate in excess of 3 months (Kelley *et al.*, 1995).

4.2 Experimental design and objectives

The aim of work in this chapter was to test whether in foetal retinal progenitor cultures a combination of EGF and bFGF could either induce rod photoreceptor differentiation or activate the expression of late neuronal markers e.g. S-Opsin, Nrl, Crx, etc. Their effect on cell proliferation and survival was also evaluated. Cells were seeded at a density of 1.0×10^4 cells/cm^2 on laminin-coated dishes and cultured with or without the addition of 10 ng/ml bFGF and 20 ng/ml EGF to the culture medium. These cultures were either maintained for 7 days with medium changes every other day until the eventual fixation and immunocytochemical analysis was undertaken, or were trypsinised daily and cells counted using a haemocytometer.

4.3 Results

Cell proliferation studies yielded broadly similar results for both the GuRt05 and GuRt09 cell lines (figures 4.2 and 4.3). Both cell lines exhibited an initial drop in numbers during the 24 h immediately following plating. The cultures then stabilised before slowly increasing in number from about day 3. The presence of EGF and bFGF increased the proliferative rate in both cell lines, though after 7 days the GuRt05 cells appeared to lose viability. This may have been due to overgrowth, although the GuRt09 cells were still in log phase growth at this time, and the cultures did not appear especially dense. The doubling time of the GuRt05 cells during log phase growth was approximately 4 days in the presence or absence of bFGF and EGF, though the growth factors appeared to initiate proliferation some 2 days earlier than cultures in medium alone. The later response of the cells cultured in the absence of growth factors could be due to the cells conditioning their own medium. The GuRt09 cells took longer to respond to bFGF and EGF, remaining constant in cell number from day 2 to 4. After this point, cells proliferated rapidly for 48h, with a doubling time of less than 2 days, before numbers eventually declined. Taken together, these results show that bFGF and

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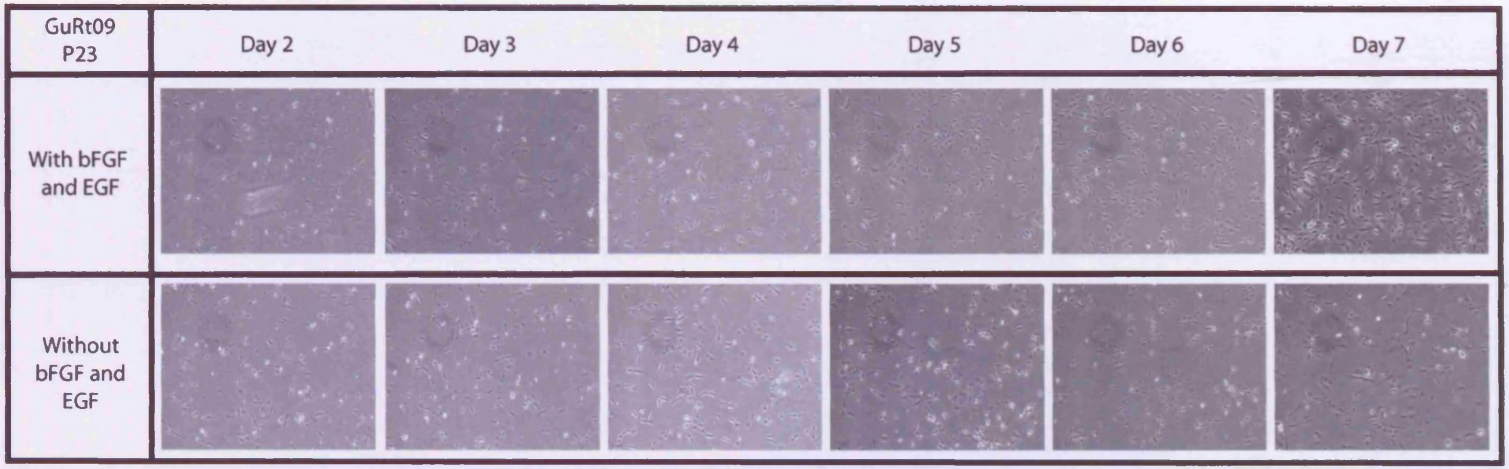

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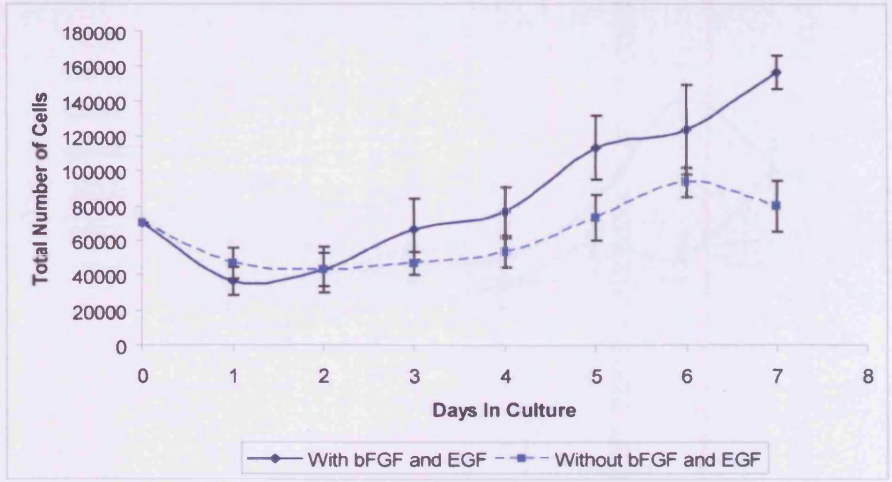

Figure 4.2 GuRt09 P23 cells cultured in the presence and absence of bFGF and EGF for 7 days
(A) Representative phase contrast images showing the GuRt09 cells on consecutive days in the presence or absence of growth factors.
(B) EGF and bFGF promoted proliferation of the immortalised human foetal retinal progenitor cells in vitro when exposed to 10ng/ml bFGF and 20ng/ml EGF. All cultures were started with 70,000 cells seeded per well (10,000 cells/cm^2). The data is presented as the mean total number of cells $\pm$ SEM.

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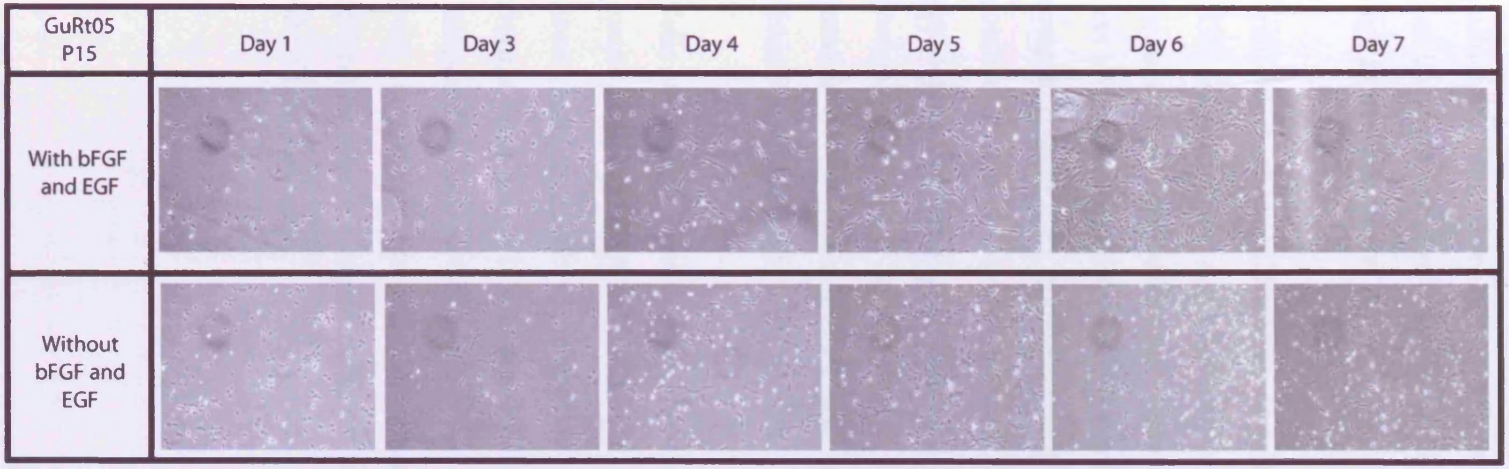

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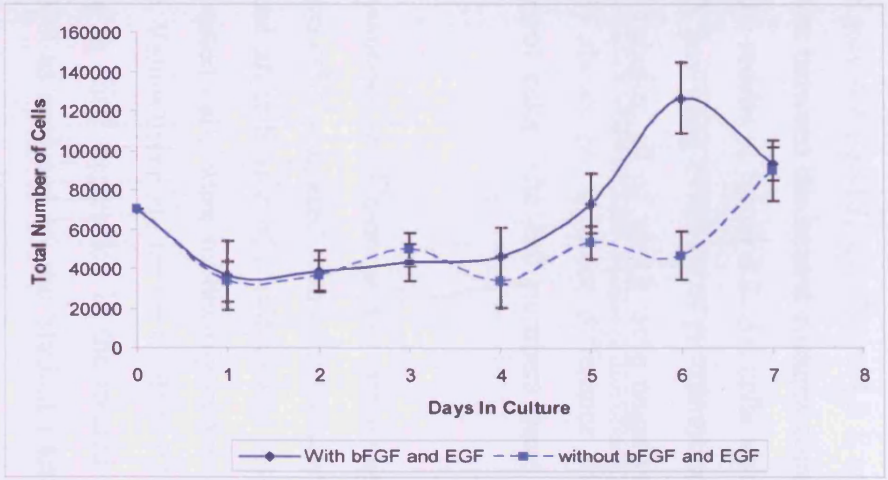

Figure 4.3 GuRt05 P15 cells cultured in the presence and absence of bFGF and EGF for 7 days
(A) Representative phase contrast images showing the GuRt05 cells on consecutive days in the presence or absence of growth factors.
(B) bFGF and EGF promoted the proliferation of the immortalised human foetal retinal progenitor cells *in vitro* when exposed to10ng/ml bFGF and 20ng/ml EGF. All cultures were started with 70,000 cells seeded per well (10,000 cells/cm^2). The data is presented as the mean total number of cells ± SEM.

EGF stimulate the proliferation of both cell lines, both of which nevertheless exhibit slow population growth when compared to many routinely cultured lines. However, caution is required in the interpretation of these results since numbers of viable cells in a culture are determined not only by proliferation rate but also by cell death. It is possible that the apparent slow growth rate of these cell lines is at least partly attributable to a high rate of apoptosis.

Immunocytochemical analysis of cells cultured in the presence or absence of bFGF and EGF did not reveal any observable differences, nor was there induction of rhodopsin expression in either of the cell lines (figure 4.4 I and J, and figure 4.5 E and F). There was a marked difference in cell numbers between the treated cultures compared to the controls (figure 4.4), consistent with the results in figure 4.2. All cells visualised were positive for the cell cycle marker Ki67, providing evidence of proliferation. Cells were also stained for Sox2, Crx and ß III Tubulin, all of which were negative. A similar analysis of GuRt05 cells (figure 4.5) shows no apparent difference in expression patterns between the treated and control cells. The cell numbers were not greatly dissimilar at this stage.

Cells were also analysed by flow cytometry to determine the percentages of Ki67 positive cells in bFGF and EGF cultured cells compared with cells cultured in medium alone. Ki67 is a cell cycle marker, and an indicator of a mitotically active cell. An average of 45% of bFGF and EGF treated cells were mitotically active (Figure 4.6) compared to 38% in the untreated cells. Values given are for the GuRt09 P23 cell line. Even though there is a slight increase in Ki67 expression in the treated cultures the difference was not significant, $p= 0.399$ as analysed by the Student t-test, where $p= <0.05$ is significant.

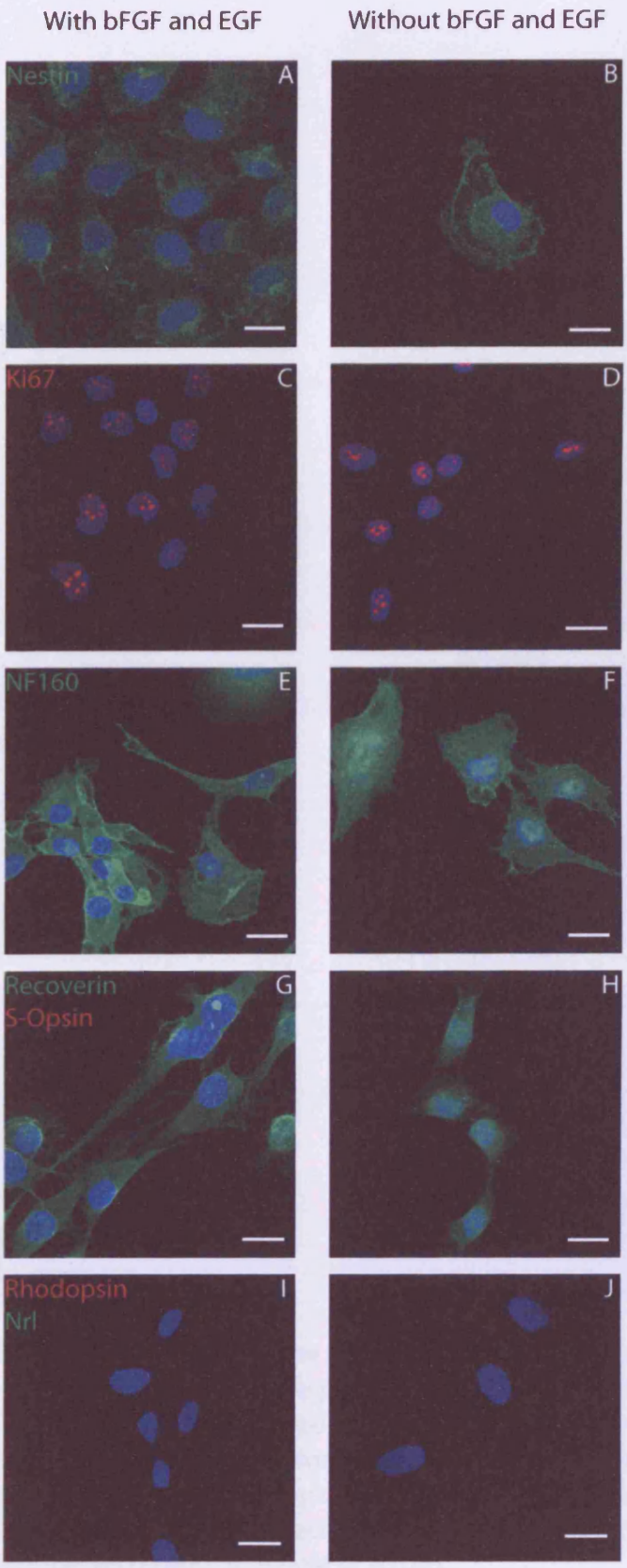

Figure 4.4 GuRt09 cells treated in the presence and absence of bFGF and EGF
GuRt09 (P19) cells were treated in the presence or absence of bFGF and EGF for 7 days at 37°C on laminin coated dishes. (A, C, E, G and I) Cells cultured in medium and 10 ng/ml bFGF and 20 ng/ml EGF for 7 days. (B, D, F, H and J) Confocal images of cells cultured in medium without added growth factors. The panels on the left show greater numbers of cells than those on the right, consistent with enhanced proliferation. Nuclei in all images were counterstained with DAPI. Scale bar = 20 μm.

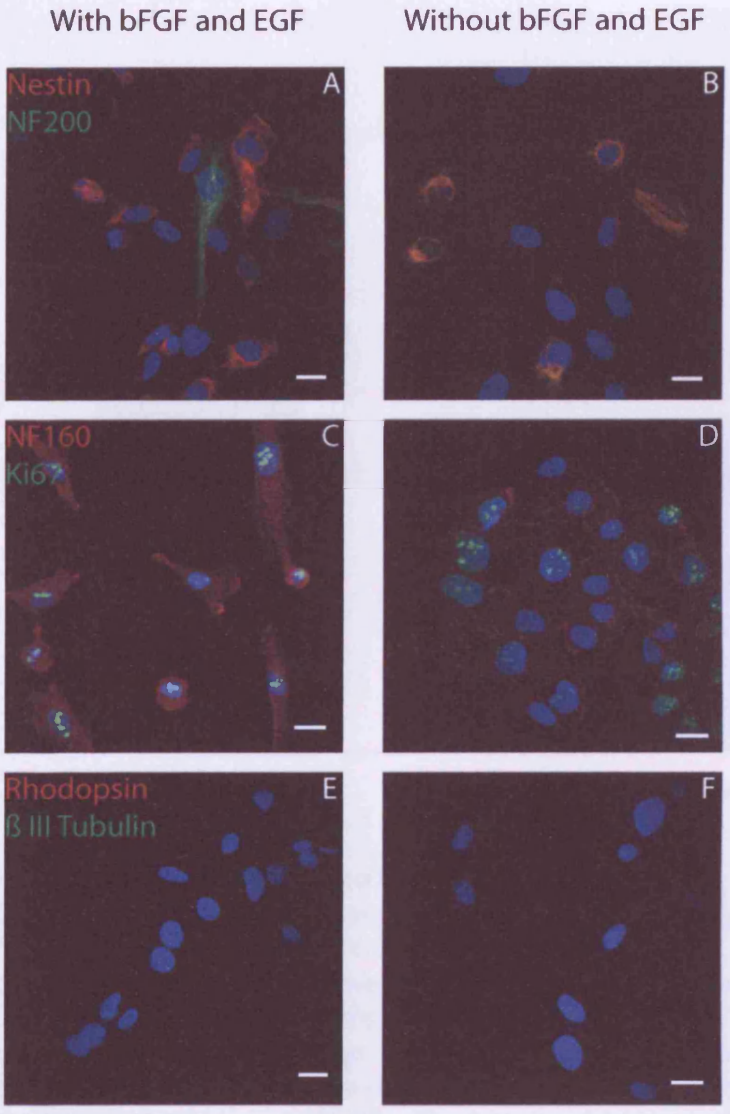

Figure 4.5 GuRt05 cells treated in the presence and absence of bFGF and EGF
GuRt05 (P15) cells were cultured in the presence or absence of bFGF and EGF for 7 days at 37˚C. (A,C and E) Cells cultured in medium and growth factors (10 ng/ml bFGF and 20 ng/ml EGF). (B, D and F) Images of cells cultured in medium alone. Cells were negative for rhodopsin, S-opsin and ß III tubulin. There was no apparent difference in expression patterns of nestin, NF200, NF160 and Ki67. All nuclei were counterstained blue with DAPI. Scale bar = 20 µm.

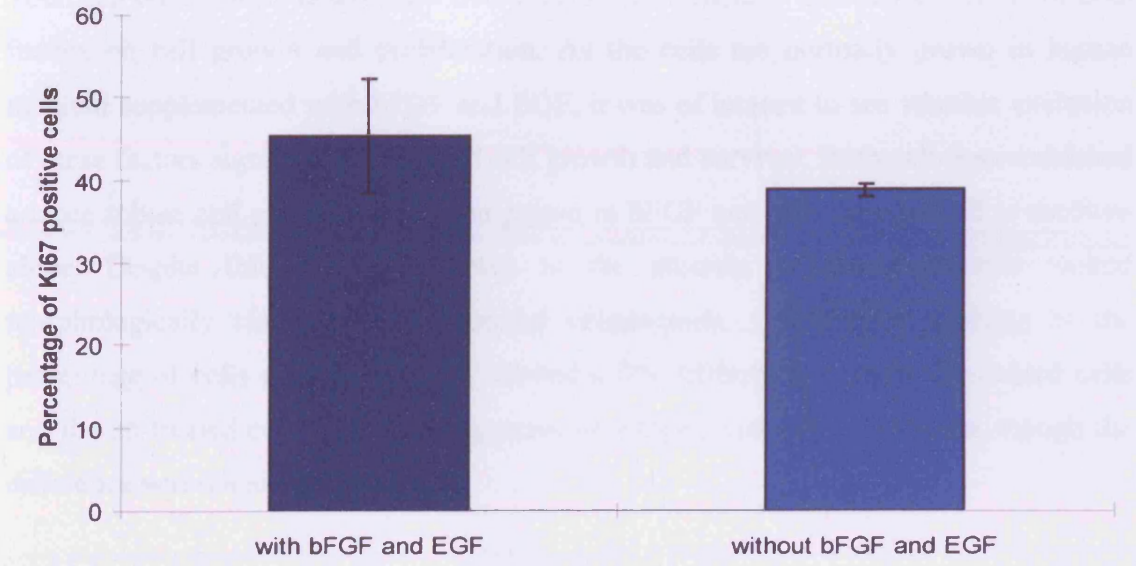

Figure 4.6 Effect of bFGF and EGF on Ki67 expression
GuRt09 cultured for 7 days were analysed via flow cytometry for the percentage of Ki67 positive cells. There is a higher percentage of mitotically active cells in cultures treated with bFGF and EGF than in those treated in medium alone. However, there is no significant difference between the cells treated with growth factors and those without, as analysed by the Student t-test ($p = 0.399$) ($n = 3$), where $p < 0.05$ is significant.

4.4 Discussion

Basic fibroblast growth factor and epidermal growth factor are well characterised mitogenic peptides that effect cell proliferation and retinal development to varying degrees. In this chapter, immortalised human foetal retinal progenitor cell lines, GuRt09 and GuRt05 were cultured in the presence or absence of bFGF and EGF at 10 ng/ml and 20 ng/ml respectively. The aim was to observe whether or not bFGF in combination with EGF would drive proliferation or differentiation, and in the case of the latter whether it would be possible to detect rhodopsin expression.

Total cell counts were determined over a period of 7 days, to quantify the effect of both factors on cell growth and proliferation. As the cells are normally grown in human medium supplemented with bFGF and EGF, it was of interest to see whether exclusion of these factors significantly affected cell growth and survival. Both cell lines exhibited a more robust cell proliferation when grown in bFGF and EGF as opposed to medium alone. Despite this the cells grown in the absence of growth factors looked morphologically similar to their treated counterparts. Quantitative analysis of the percentage of cells expressing Ki67 showed a 7% difference between the treated cells and the un-treated cells which is suggestive of a higher rate of proliferation, though the difference was not significant.

A link has been established between the age of a retinal cell culture and its responsiveness to FGF. When cells are taken from late embryonic to early postnatal retina, higher concentrations of the mitogens are required compared to early embryonic cultures, where maximal effects are observed with the lowest of mitogen concentrations (Lillien and Cepko, 1992). This seems to imply that foetal cells that are developmentally mid-way between these time points may require higher concentrations of growth factors to elicit maximal effects. However, the experiments were carried out at concentrations for both bFGF and EGF found to be effective in previous studies (Hicks and Courtois, 1992b). The possibility remains that with higher concentrations of both growth factors there could have been significantly higher proliferation rates in the treated cultures. These data indicate that the combination of bFGF and EGF functions primarily to prevent cells from dying, and thereby promotes cell survival in the treated cultures.

As bFGF has been shown to induce opsin expression (Hicks and Courtois 1992b), it was possible that this might occur in the foetal retinal progenitor cell lines. However, the cells continued to express the same selection of markers with and without bFGF and EGF, with no discernable difference in expression patterns. It is known that the age of a cell is of utmost importance along with the factors added to the culture medium. With regards to the work carried out by Hicks and Courtois (1992), they found increased opsin expression in the presence of aFGF and bFGF, although the age of the cells had a contributing role. The cells were derived from postnatal rat retinae, and as a majority of photoreceptors develop postnatally in rodents these cells probably had far fewer cell fate decisions to undertake before differentiating into rod photoreceptors. It therefore seems likely that the immortalised human foetal progenitor cell lines may be far less capable of responding in the same way.

In conclusion, EGF and bFGF were found to have mitogenic effects on the immortalised retinal progenitor cell lines, but to have no effect on the differentiation of these cell lines. It could be that the concentrations of both factors were inappropriate with regard to the age of the cell lines. Thus, establishing a cocktail of trophic factors in the cellular microenvironment itself is a major challenge, and knowing the best possible developmental time point at which to model the environment is quite another.

Chapter 5

The effect of foetal bovine serum on immortalised retinal progenitor cells

Chapter 5

The effect of foetal bovine serum on immortalised retinal progenitor cells

5.1 Introduction

Foetal Bovine Serum (FBS) is routinely used when maintaining cell lines in culture, this is because of the plethora of trophic factors present within the serum that are required for *in vitro* culturing systems. The withdrawal of bFGF and addition of FBS to the medium of some human retinal progenitor cells (taken from the same time point as the immortalised human foetal retinal progenitor cells) has led to the differentiation of these cells (Yang *et al.*, 2002a). Yang and colleagues (2002b) began to explore the use of FBS as a means to differentiate rat retinal progenitor cells, and concluded that in the presence of 10% FBS cells began to express early neuronal marker such as ß III Tubulin, and more mature markers such as rhodopsin and S-opsin, suggesting that the *in vitro* microenvironment, containing FBS, induced the cells to mature. FBS has been shown to induce differentiation not only towards a neuronal phenotype but also towards cardiac differentiation in human embryonic stem cells (Bettiol *et al.*, 2007). However these findings are at odds with other studies where the addition of FBS to human foetal retinal cells led to continuous cell proliferation without any evidence of differentiation (Kelley *et al.*, 1995). Therefore there is still a great deal of ambiguity as to the trophic effects FBS has with respect to the differentiation of retinal progenitor cells towards a neuronal lineage.

5.2 Experimental design and objectives

The aim of work in this chapter was to observe whether or not FBS has any effect on differentiation of the immortalised human foetal retinal progenitor cells lines, and could possibly activate photoreceptor differentiation. Cells were seeded at a density of 1.0 x 10^4 cells/ cm^2 on laminin-coated dishes and cultured at 37°C, in DMEM:F-12 medium alone, or supplemented with 5% FBS or 10% FBS. Cells were incubated for 7 days with medium changes every other day. At the end of this period cells were fixed and immunoassayed for various markers and imaged using a confocal microscope.

5.3 Results

Growth in the presence of FBS led to far greater numbers of cells per dish compared to the cells cultured in medium alone. However, there was no obvious difference in expression patterns for nestin and NF200 (figures 5.1 and 5.2) at all three conditions. Nestin did not seem to decline, as might have been expected if cells were beginning to differentiate, and there was no apparent difference in the expression patterns of NF200 in both cell lines.

Following 7 days in culture, the GuRt09 and GuRt05 cell lines both expressed NF160 and Ki67 (figure 5.3) with no clear difference in expression patterns for all three conditions investigated. The increase in cell numbers is evident in figure 5.3 C and F compared to the cells cultured in the plain medium alone (figure 5.3 A and D).

The expression of recoverin was also observed in all three conditions, with no apparent difference in expression patterns (figure 5.4), however S-opsin expression was not detected in any of the conditions tested, for either of the clonal cell lines. Therefore cells had not begun to differentiate towards a cone photoreceptor lineage.

In summary, the cells did not respond as one would expect of multipotent retinal progenitors, and induction of rhodopsin expression was not observed (figure 5.5 G-L) for either of the cell lines in any of three conditions tested. Rhodopsin kinase, a protein involved in the termination of phototransduction in rod and cone photoreceptors, was nevertheless positive in the control cells and in the 5% and 10% FBS treated cultures. The expression of Nrl and Crx transcription factors was not detected via immunocytochemistry in either cell line.

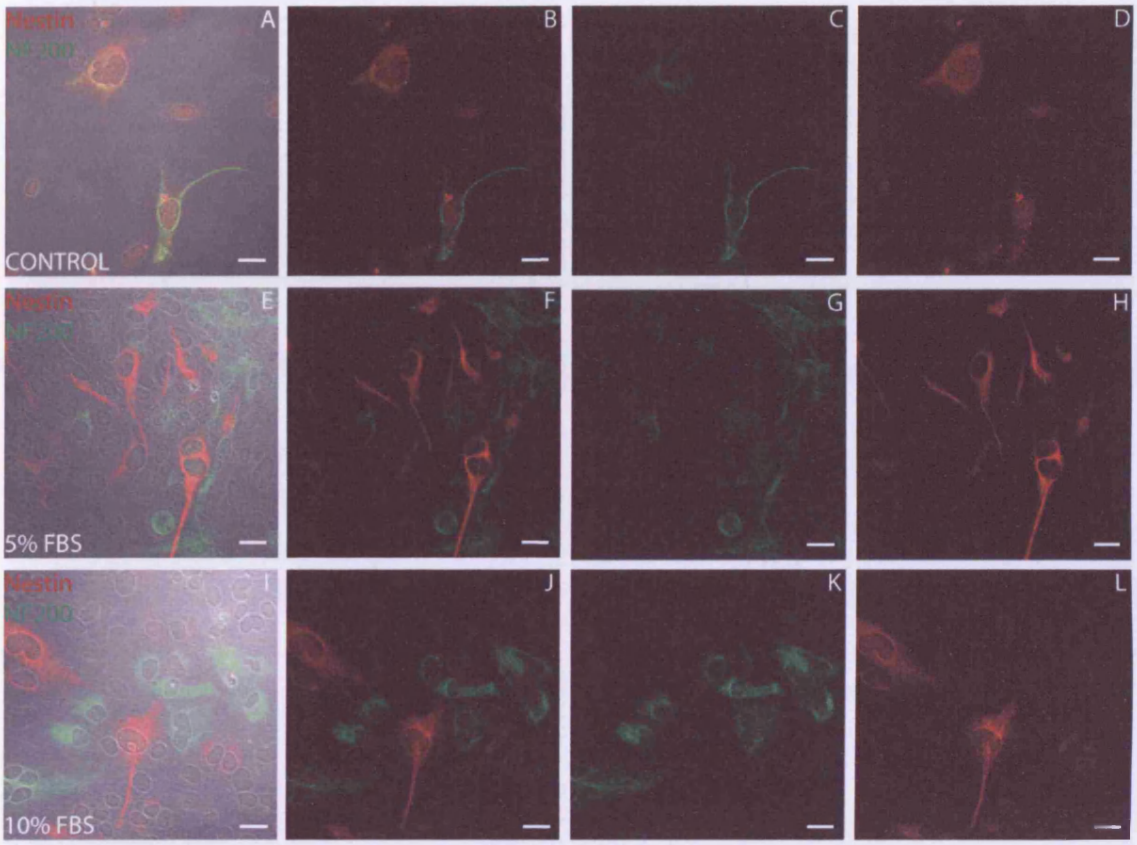

Figure 5.1 Expression of nestin and NF200 in the presence of FBS for GuRt09 cell line
GuRt09 (P24) cells cultured in media with the addition of 5% FBS or 10% FBS for 7 days prior to fixation and staining. (A-D) Control cells were cultured in medium only for 7 days. (E-H) Cells cultured in 5% FBS were positive for nestin and NF200 markers. (I-L) Cells cultured in 10% FBS were also positive for nestin and NF200. There was no apparent difference in expression patterns of these two markers. However, as FBS contains a plethora of growth factors the number of cells increased for those cells cultured in FBS compared to the control cultures. Scale bar = 20 μm.

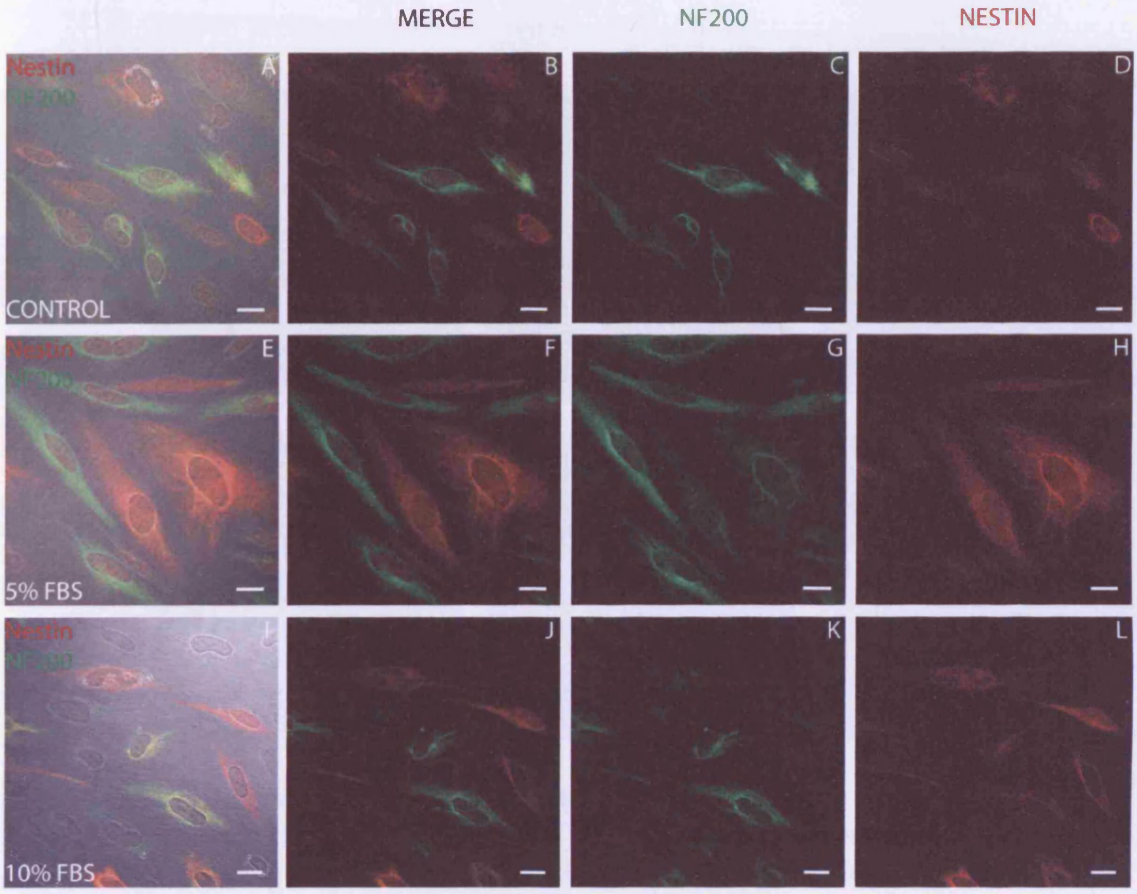

Figure 5.2 Expression of nestin and NF200 in the presence of FBS for GuRt05 cell line
GuRt05 (P24) cells cultured in media with the addition of 5% FBS or 10% FBS for 7 days prior to fixation and staining. (A-D) Control cells were cultured in medium only for 7 days. (E-H) Cells cultured in 5% FBS were positive for nestin and NF200. (I-L) Cells cultured in 10% FBS were also positive for nestin and NF200. There was no apparent difference in expression patterns of these two particular markers. However, as FBS contains a plethora of growth factors the number of cells increased for those cells cultured in FBS compared to the control cultures. It is also interesting to note that cells that are positive for NF200 (C,G,K) tend to express lower levels of nestin (D,H,L) and vice versa. Scale bar = 20 μm.

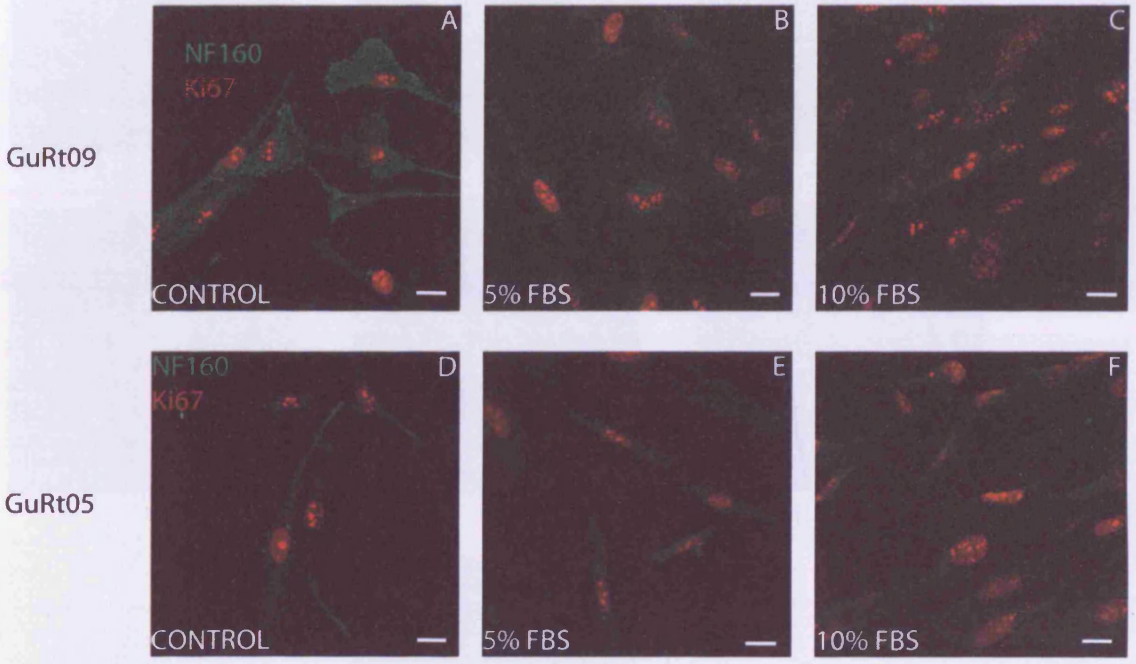

Figure 5.3 Expression of NF160 and Ki67 in GuRt09 and GuRt05 cells treated with FBS
Confocal images of the immortalised retinal progenitor cell lines treated for 7 days with 5% FBS or 10% FBS at 37˚C on laminin-coated dishes for 7 days. (A,B,C) Images of GuRt09 cells in (A) control medium without FBS, (B) 5% FBS or (C) 10% FBS. Markers for NF160 and Ki67 were positive at all three conditions. (D,E,F) Confocal images of GuRt05 cells in (D) control medium, (E) 5% FBS or (F) 10% FBS. All cells were positive for NF160 and Ki67. There was no apparent difference in expression patterns of NF160 and Ki67 between all cell cultures and between cell lines. Scale bar= 20 µm.

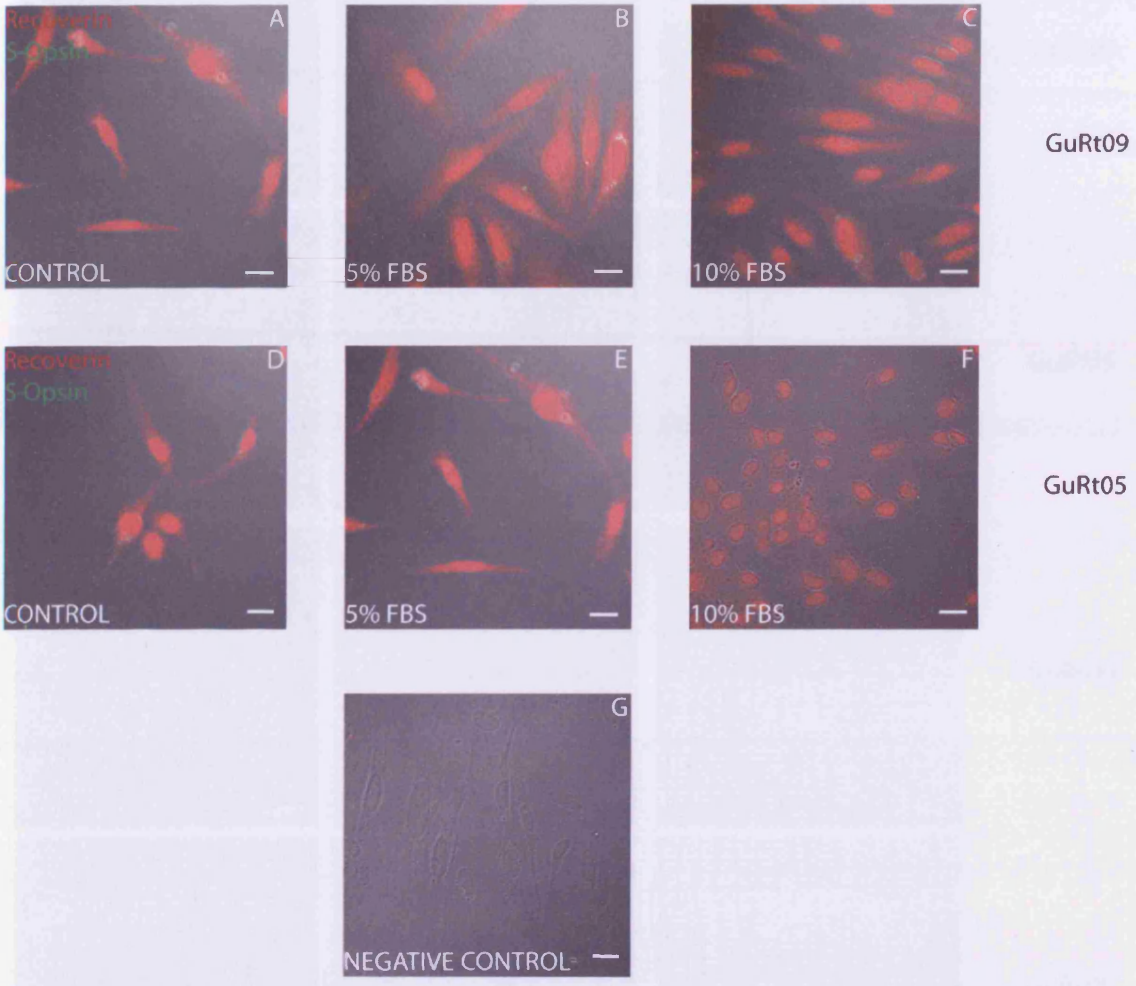

Figure 5.4 Recoverin and S-Opsin expression in FBS treated cell cultures
GuRt09 and GuRt05 cell lines were cultured in the presence of medium, 5% FBS or 10% FBS for 7 days and subsequently fixed and stained for recoverin and S-Opsin. (A,B,C) GuRt09 cells were positive for recoverin expression at all three conditions, however S-Opsin was undetected. (D,E,F) The expression patterns for recoverin were similar at all three conditions in GuRt05 cells, the only visible difference was that there were more cells present in cells cultured with 10% FBS. (G) A negative control minus the primary antibodies showed no non-specific staining.
Scale bar = 20 µm.

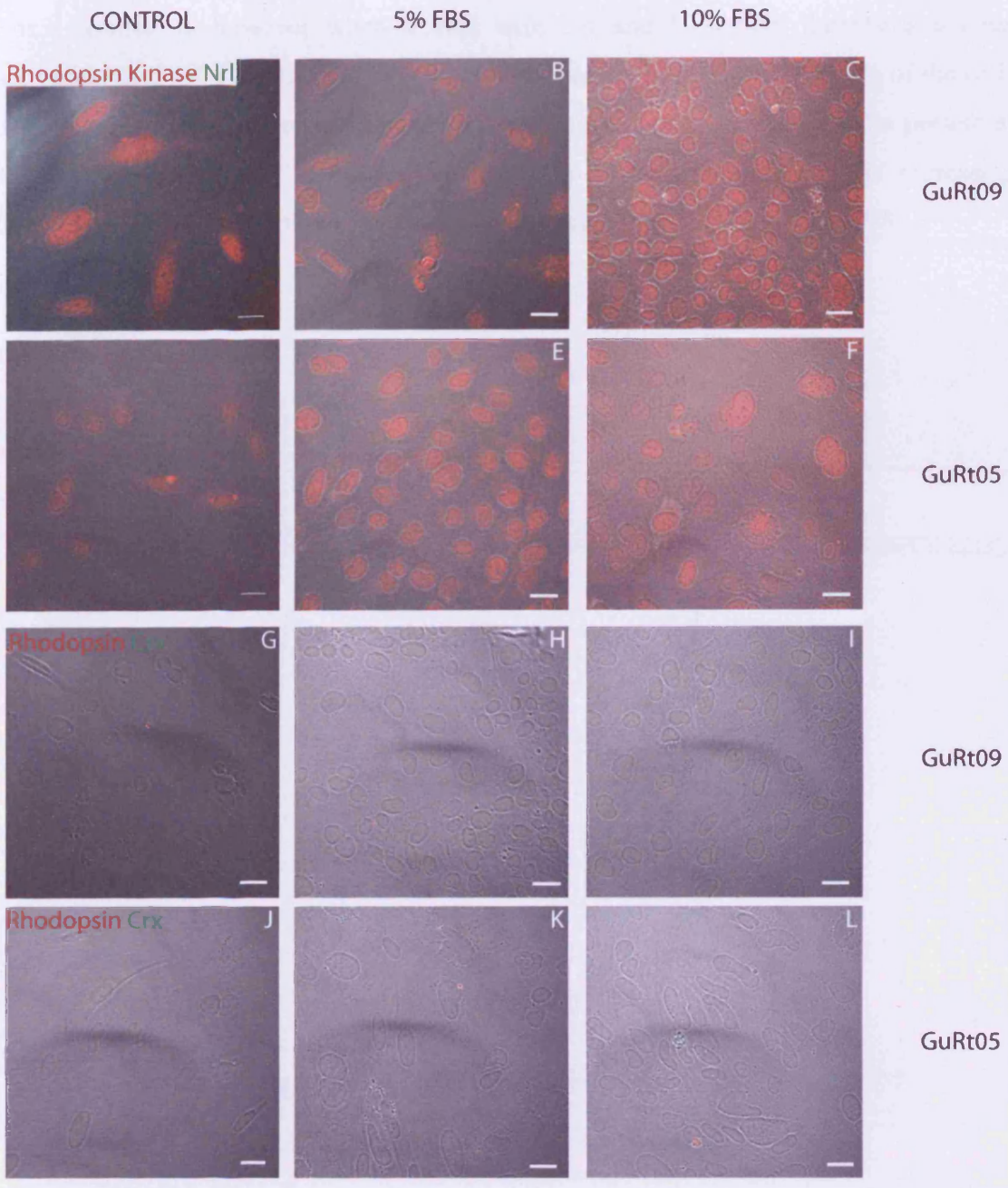

Figure 5.5 Expression of rhodopsin kinase, rhodopsin, Nrl and Crx in FBS treated cultures
GuRt09 and GuRt05 cells were cultured in medium alone, or supplemented with 5% or 10% FBS for 7 days. (A-F) Rhodopsin kinase expression was detected in all three conditions for both cell lines with no apparent difference in expression patterns. Nrl expression was not detectable. (G-L) Rhodopsin and Crx were also immunostained but neither marker was detected in any of the conditions tested. Scale bar = 20 μm.

Both cell lines were found to be immunoreactive for the ß III Tubulin antigen which is a marker for immature neurons as well as ganglion cells (figures 5.6 and 5.7). In the cells cultured in medium without FBS there was no detection of ß III Tubulin expression or Sox2 expression, however when treated with 5% and 10% FBS there was a clear activation of ß III Tubulin expression which appeared to be present in 100% of the cells. Therefore, at both concentration of FBS, even though there were more cells present at 10% FBS than at 5% FBS there was no difference in the percentage of cells expressing ß III Tubulin. There was no detection of Sox2 expression at 5% and 10% FBS.

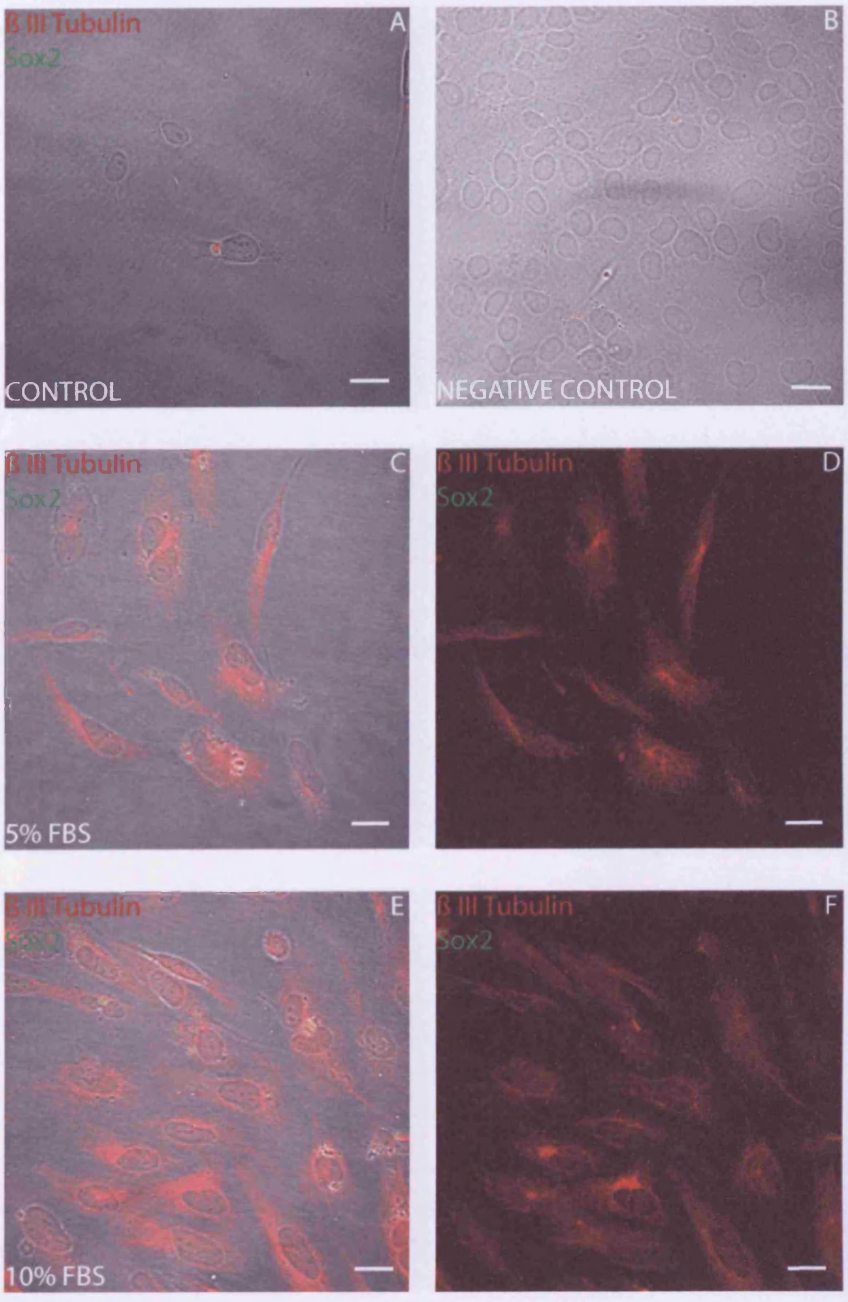

Figure 5.6 Activation of ß III tubulin expression in GuRt09 cells under the influence of FBS
GuRt09 (P24) cells were cultured in 5% FBS or 10% FBS for 7 days. (C-F) This resulted in the detection of ß III tubulin expression in both culture conditions, which was absent from the cells in the control culture (A). (B) Image of negative control, where the primary antibodies were omitted from the staining protocol in order to observe any non-specific staining. Sox2 was undetectable in control and treated cell cultures. Scale bar = 20 µm.

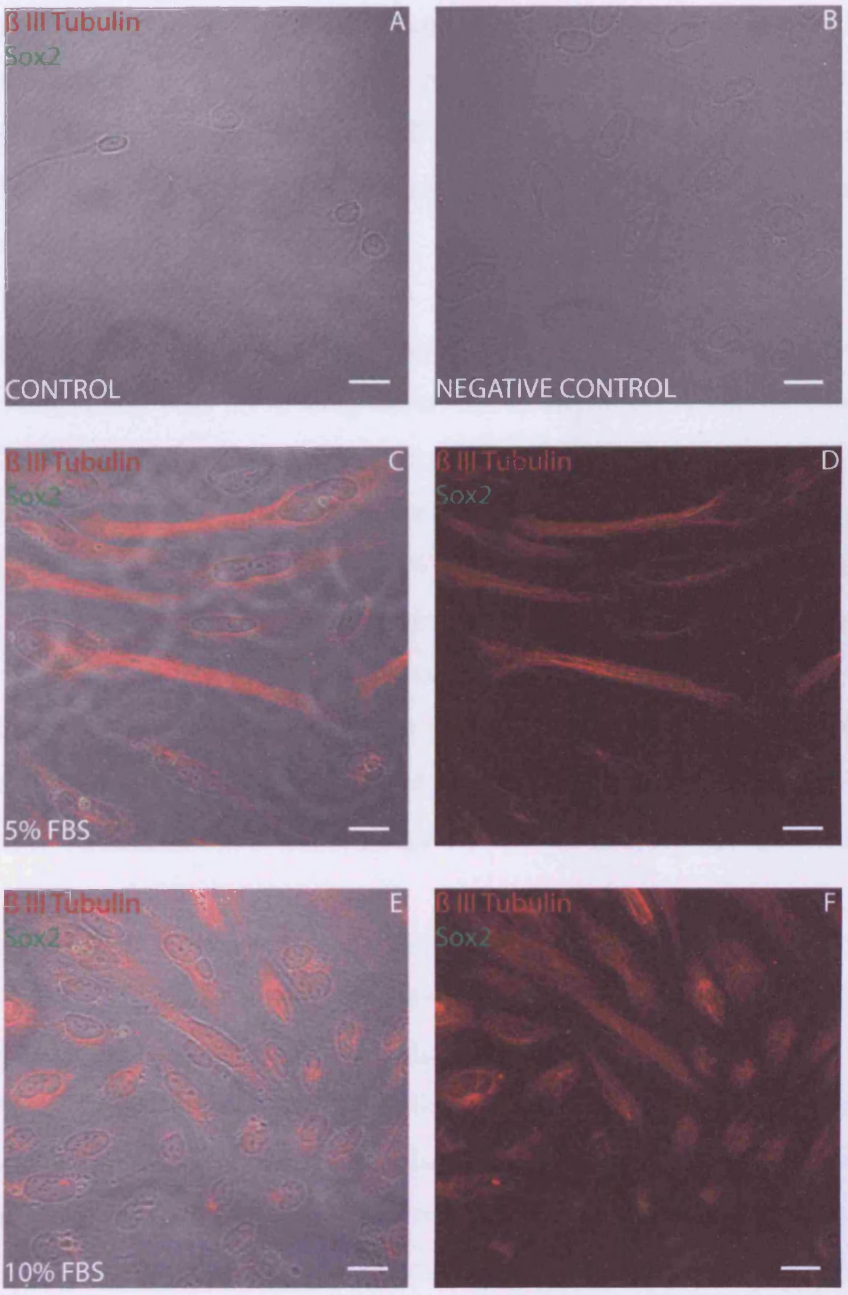

Figure 5.7 Activation of ß III tubulin expression in GuRt05 cells under the influence of FBS
GuRt05 (P24) cells were cultured in 5% FBS or 10% FBS for 7 days. (C-F) This resulted in the detection of ß III tubulin expression in both culture conditions, which was absent from the cells in the control culture (A). (B) Image of a negative control, where the primary antibodies were omitted from the staining protocol in order to observe any non-specific staining. Sox2 was not detected in control and treated cell cultures. Scale bar = 20 µm.

5.4 Discussion

FBS has been shown to effect cell differentiation in human and mouse retinal progenitor cells and to induce ß III Tubulin, S-Opsin and rhodopsin expression in certain cases. In this chapter, immortalised human foetal retinal progenitor cell lines, GuRt09 and GuRt05 were cultured in the presence of 5% FBS, 10% FBS or plain medium to observe whether or not FBS could induce differentiation in the cell lines, with a particular interest in rhodopsin induction.

Immunocytochemical analysis revealed expression of the same selection of markers with and without FBS, with no striking difference in expression patterns. Rhodopsin, S-opsin, Sox2, Nrl and Crx expression were not detected in the control or FBS-treated cells. However, with regard to the immature neuronal marker and ganglion cell marker, ß III Tubulin, both cell lines cultured in 5% FBS and 10% FBS expressed this protein. This is the first time that ß III Tubulin expression had been detected in these cell lines. In Chapter 3, ß III Tubulin mRNA expression was detected via RT-PCR but not with immunocytochemistry. This implies that these cells may have the potential to differentiate towards a ganglion cell fate and is consistent with the additional expression of NF160 and NF200, both of which are expressed in the axons of ganglion cells.

Cell differentiation in the vertebrate retina is initiated by the sequential production of cell types in a well defined histogenetic order (Marquardt and Gruss, 2002). Retinal neurogenesis begins with the differentiation of ganglion cells and the behaviour exhibited by the GuRt09 and GuRt05 cell lines seems to comply with this histogenetic order. Thus the cells are capable of committing towards a particular lineage even though they are not fully differentiated, as the expression of nestin would be expected to diminish. It may be that the cells would only terminate nestin expression following a longer culture period in the presence of FBS.

This chapter has shown that the immortalised human foetal retinal progenitor cells are capable of responding to FBS in the same manner as unimmortalised human foetal retinal (Yang *et al.*, 2002a) by beginning to differentiate towards a ganglion cell fate. Even though the cells did not respond with the induction of rhodopsin expression, perhaps more specific trophic factors especially involved in the induction of rhodopsin expression will induce these cell lines to differentiate further. This will be explored in more detail in subsequent chapters.

Chapter 6
Retinoic acid and the immortalised retinal progenitor cell lines

Chapter 6

Retinoic acid and the immortalised retinal progenitor cell lines

6.1 Introduction

Vitamin A plays a crucial role in the development of the Central Nervous System (CNS) and is required at specific levels in order for development to occur properly (Maden *et al.*, 1992). Vitamin A is also an important component of the visual system. The aldehyde form of the vitamin (retinal or retinaldehyde) is the chromophore of all opsin pigments (Hyatt and Dowling, 1997). It plays an essential role in the phototransduction pathway, whereby the 11-*cis* retinal isomer becomes activated by light and is converted to the all-*trans* form, initiating excitation of the photoreceptor cells. Due to the essential role retinal plays in vision, it has been discovered that the acid form of vitamin A, retinoic acid (RA), is critical for early eye development and aids photoreceptor differentiation by the activation of a large family of transcription factors (Dräger and McCaffery, 1997).

6.1.1 Retinoic acid signalling pathway

When RA enters a cell, it binds to nuclear receptors that act as ligand-dependent transcription factors (Mangelsdorf and Evans, 1995) that subsequently go on to activate or inhibit gene transcription. There are several forms of RA and two families of receptors, the RARs (α, ß, and γ and their isoforms α1, α2, ß1 to ß4, and γ1 and γ2) and RXRs (α, ß, and γ) (Kastner *et al.*, 1994) which are activated by the different forms of retinoic acid. The retinoic acid receptors (RARs) bind to both all-*trans*-RA and 9-*cis*-RA, whereas the retinoid X receptors (RXRs) only bind to 9-*cis*-RA (Klaassen *et al.*, 1999). For example, all-*trans*-RA is the ligand for the RAR family of receptors, and once bound the receptor forms heterodimers with RXR, which in turn acts as a transcription factor targeting particular regulatory elements of key genes. The regulatory regions of the target gene are known as the RA-responsive DNA elements (RAREs), and when bound to the RARE the active RAR/RXR can then control gene expression and induce or repress the transcription and eventual translation of the target protein.

6.1.2 Retinoic acid and ocular development

The role of retinoic acid in ocular development was discovered using transgenic indicator mice (Balkan *et al.*, 1992). A recombinant reporter gene comprised of three copies of the RARE sequence, originally from the RARß-2 promoter, and the herpes simplex virus thymidine kinase promoter coupled to a bacterial ß-galactosidase reporter gene was created to detect activated RA *in vivo* (figure 6.1). RARE sequences from the RARß-2 promoter were used in this construct due to their increased sensitivity towards the presence of RA-bound RAR receptors. The injection of this recombinant ß-galactosidase reporter into mouse ova produced a visible method to identify areas in which activated RXR and RAR receptors were situated and hence where retinoic acid signalling was most prevalent during embryonic development. At day 12.5 in gestation, the embryos exhibited two clear regions containing the highest levels of ß-galactosidase activity, the first area being the dorsal aspect of the embryo, the second being the eye. In comparative studies between control embryos and RA-treated embryos, both displayed high levels of ß-galactosidase activity in the neural retina, lens, and to a smaller extent in the pigmented retinal layer.

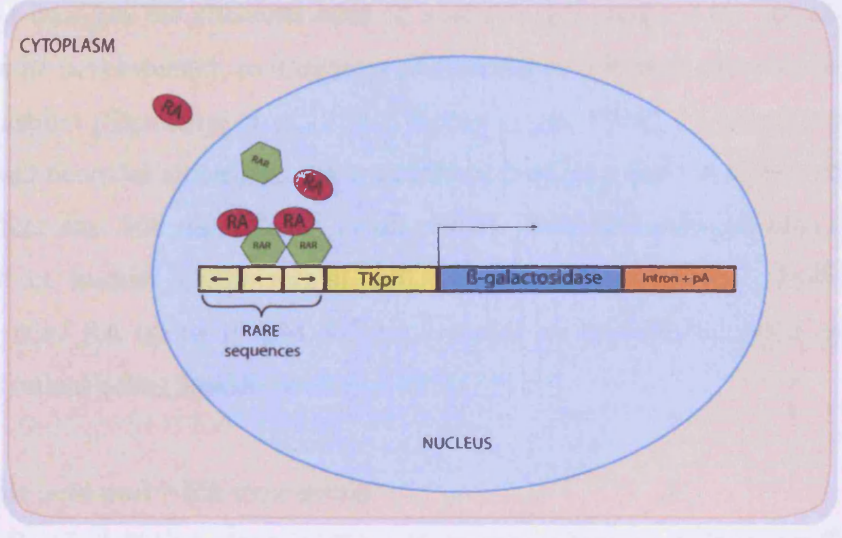

Figure 6.1 Schematic representation of the recombinant reporter gene used to detect activated RARs in transgenic mice

In the transgenic mice, expression of the reporter gene, ß-galactosidase, serves as an indicator for activated receptors. These receptors are activated either by endogenous Retinoic Acid (RA) or when females carrying transgenic mice are gavage-fed RA. A constitutively active RAR vector is produced comprising of three copies of the RA response DNA elements (RARE), that are coupled together with the herpes simplex virus thymidine kinase promoter (TKpr), the reporter gene, and finally an SV40 intron plus the poly(A) addition signal. (Adapted from Balkan *et al.*, 1992).

This study revealed the existence of activated RARs and RXRs within these regions, and consequently the existence of natural RA interactions during eye development *in vivo*.

6.1.3 Retinoic acid and rod photoreceptor development

The vital role of RA in eye morphogenesis has also been confirmed by modifying the levels of RA during early eye development in zebrafish (Hyatt *et al.*, 1996). This study highlighted the role RA plays in the induction of rod photoreceptor differentiation and survival *in vivo*. In RA treated embryos, *in situ* hybridisation for rhodopsin mRNA showed an increased level of expression in the ventral region of the eye as the experiment progressed. This effect was not observed in retinol- or retinal-treated embryos. Immunohistochemical staining for rhodopsin correlated with the pattern of rhodopsin mRNA expression. Conversely, treatment with Citral, an inhibitor of RA synthesis, in the ventral region of the retina, resulted in a dose-dependent inhibition of rhodopsin expression. It has also been found that RA acts upon a post-mitotic population to induce rhodopsin expression (Wallace and Jensen 1999; Hyatt *et al.*, 1996).

Retinoic acid thus has the characteristics of a morphogen, namely the ability to drive the pattern of tissue development, as it induces photoreceptor differentiation *in vitro*, in a dose-dependent fashion (Stenkamp *et al.*, 1993; Kelley *et al.*, 1994). In dissociated cells from embryonic and neonatal rat retinae, the concentration of all-*trans*-RA demonstrated to have a proven effect was 500 nM (Kelley *et al.*, 1994). This concentration also produced the same effect in human foetal retinal cell cultures (Kelley *et al.*, 1995). However, concentrations of RA up to 10 μM did not generate an appreciable effect on embryonic chick neural retinal cells (Stenkamp *et al.*, 1993).

6.1.4 Retinoic acid and NRL expression

The Neural Retina Leucine zipper (NRL) transcription factor has been implicated in the development and differentiation of rod photoreceptors by activating various rod specific genes. Nrl $^{-/-}$ null mutant mice lack any rod photoreceptor function and rhodopsin immunoreactivity (Mears *et al.*, 2001), which demonstrates the importance of NRL in functional rod photoreceptor formation. Importantly, a link has been identified between RA and the RAREs situated within the NRL promoter region (Khanna *et al.*, 2006), suggesting that retinoic acid signalling can regulate NRL expression via the RARE, and therefore

identifies a RA-signalling pathway driving rod photoreceptor determination and differentiation.

6.2 Experimental design and objectives

The purpose of the experiments described in this chapter was to examine the effects of all-*trans*-retinoic acid (RA) on the retinal progenitor cell lines and to observe any differentiation patterns. In particular, we were especially interested in any differentiation towards rhodopsin expression and rod photoreceptor phenotype. According to the studies conducted by Kelley *et al.*, (1994), 500 nM of all-*trans*-RA was determined to be the optimal concentration for photoreceptor induction in rat and human retinal cells, therefore three different concentrations of RA were investigated; 500 nM, 1 μM and 3 μM. Human foetal immortalised retinal progenitor cells were seeded onto a series of laminin-coated 35-mm tissue culture dishes at a density of 1.0 x 10^4 cells/cm^2. DMEM:F12 minus L-Glutamine was supplemented with all-*trans*-RA, which was diluted to the appropriate concentration in the culture medium just prior to use. In parallel with the experiment, a vehicle control was conducted whereby a volume of DMSO equivalent to that used in the highest RA treatment was added to the cells (4.8 μl) in lieu of all-*trans*-RA. The experiment was performed for a duration of three days with daily medium changes. At this point the cultures were fixed.

During the course of the experiment it was apparent that the cells were unable to survive for prolonged periods in the presence of retinoic acid treatment alone. Therefore a different method was also employed in order to sustain a greater number of cells for analysis. Both cell lines were cultured with the same concentrations of RA but with the addition of 3% charcoal/dextran treated foetal bovine serum (FBS) (Hyclone) added to the culture medium. FBS was used to increase the survival rate of the cells, however it was essential that the FBS itself did not induce any effect. Charcoal/dextran treatment is used to lower the level of trophic factors present within serum and should therefore minimise effects on the cells other than enhancing survival. After 7 days of all-*trans*-RA treatment in the presence of 3% charcoal/dextran treated FBS, cells were fixed and immunoassayed. All experiments in this chapter were repeated four times. As inter-experimental variability was minimal, results of representative experiments are shown to simplify data presentation.

6.3 Results

Figure 6.2 shows phase-contrast images of the GuRt05 cells grown for 3 days at 37 °C in the presence of either DMSO vehicle control (Fig. 6.2A), 500 nM all-*trans*-RA (Fig. 6.2B), 1 μM all-*trans*-RA (Fig. 6.2C) or 3 μM all-*trans*-RA (Fig. 6.2D). Initial observations showed that all cultures were unable to survive for the initial seven day period in the presence of all-*trans*-RA. Only the cells cultured in the presence of the vehicle control exhibited sufficient cell survival to enable further analysis, therefore the experiment was shortened from seven to three days. Unlike the neurite outgrowth observed by Kelley *et al.*, (1994), the cells did not exhibit rounded cell bodies with long branching neurites, but retained a similar morphology to the vehicle control cells. With respect to the 3 μM treated cultures, some cells had apparent shrunken cytoplasms that resembled those of dying cells. At no point in the experiment did cells exhibit characteristic photoreceptor morphology

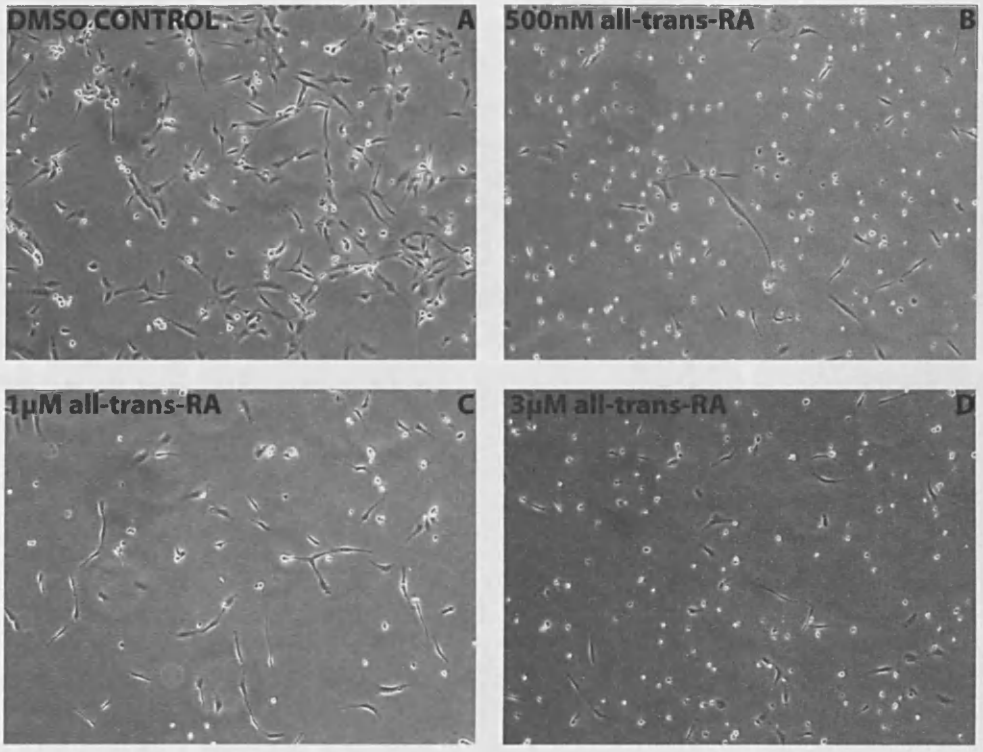

Figure 6.2 All-*trans*-Retinoic Acid (RA) treated GuRt05 cells incubated at 37˚C for 3 days
Phase-contrast images GuRt05 (P22) cells after 3 days of treatment with all-*trans*-retinoic acid at varying concentrations (A) DMSO vehicle control, (B) 500 nM all-*trans*-RA, (C) 1 μM all-*trans*-RA and (D) 3 μM all-*trans*-RA treated cells. Cells cultured with all-trans-RA showed a dramatic decrease in cell numbers compared with the vehicle control. At this time point the cells were fixed and immunocytochemical analysis was completed.

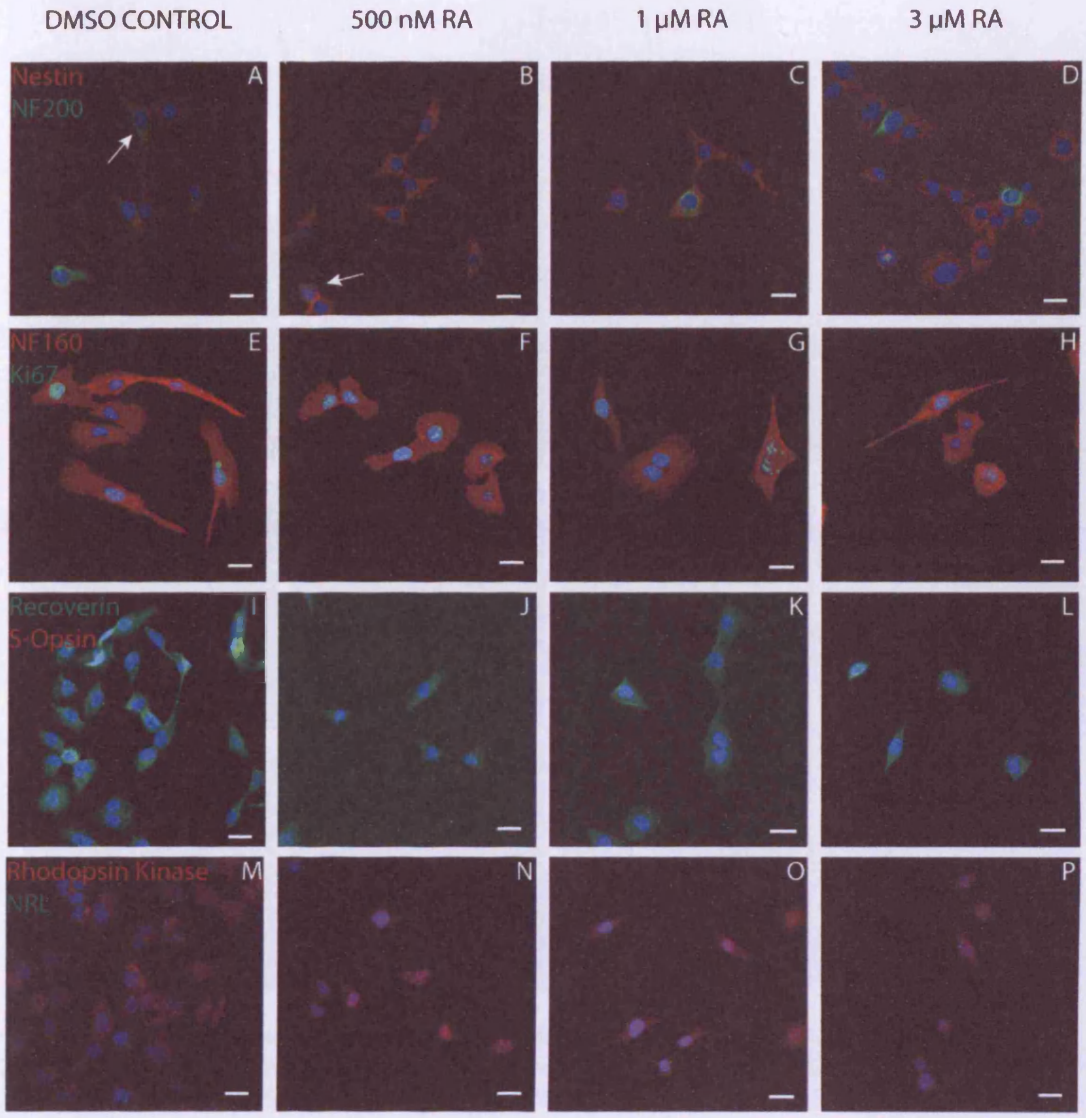

Figure 6.3 Confocal image analysis of the immunocytochemical expression patterns of GuRt09 cell line treated with different concentrations of all-*trans*-RA for 3 days.
GuRt09 (P28) cells were treated with 0.5-3.0 µM all-*trans*-RA for 3 days at 37°C. (A-D) All cells were positive for nestin with a small subset expressing NF200. (E-H) All cells were positive for NF160 and Ki67 antigens. Regardless of the high levels of dying cells, the surviving cells were still proliferating. (I-L) All cells viewed were positive for recoverin but there was no positive staining for S-opsin. (M-P) Even though NRL has been shown to be up-regulated in the presence of RA this was not the case for this cell line. However, they still remained positive for rhodopsin kinase. Compared to the DMSO vehicle control the all-*trans*-RA treated cells showed a dramatic loss in cell numbers. Nuclei are counterstained with DAPI and are blue in all images. Scale bar= 20 µm.

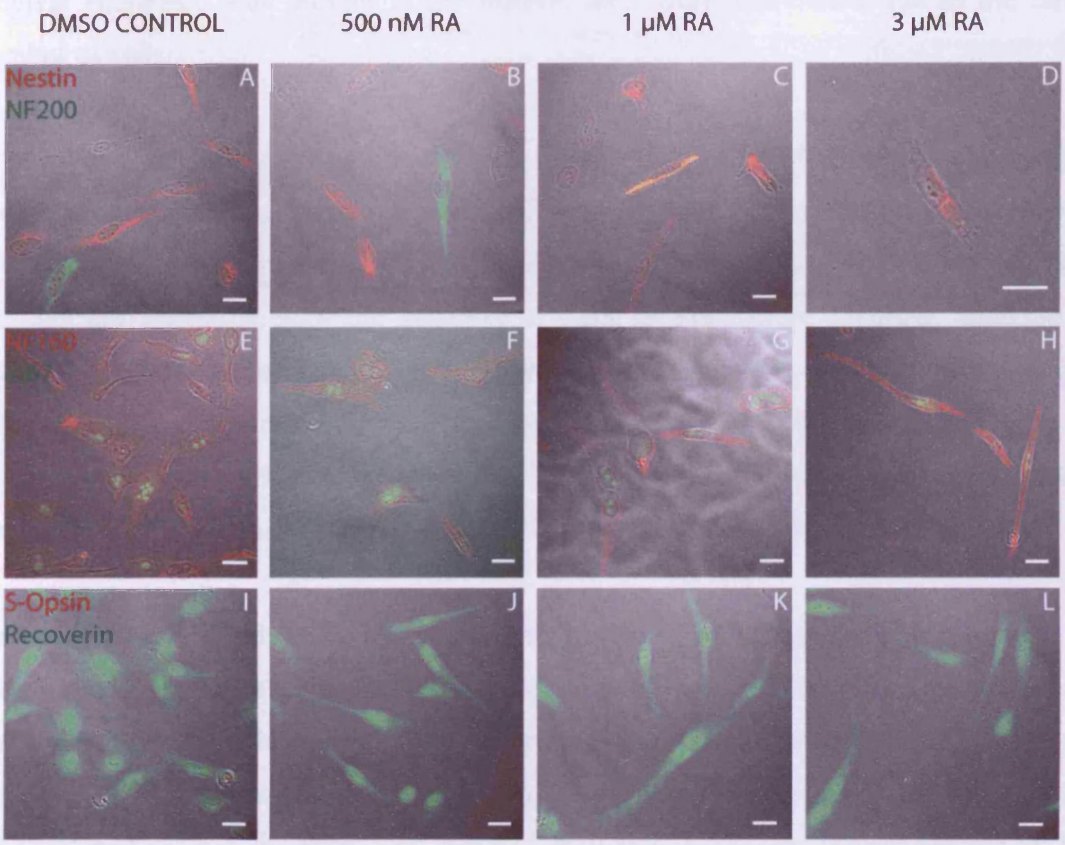

Figure 6.4 Expression patterns of GuRt05 cell line treated with all-*trans*-RA for 3 days
GuRt05 (P24) cells were treated with different concentrations of all-*trans*-RA for a duration of 3 days. (A-D) Cells were positive for nestin with a small subset expressing NF200. (E-H) All cells were positive for NF160 and Ki67 antigens. (I-L) All cells viewed were positive for Recoverin but there was no positive staining for S-Opsin. Cells were positive for rhodopsin kinase (data not shown) and were negative for rhodopsin, Crx and Nrl. Scale bar= 20 µm.

After 3 days treatment with all-*trans*-RA, GuRt09 (P28) (figure 6.3) and GuRt05 (P24) (figure 6.4) showed no change in the expression patterns of the proteins under examination. Results indicated that the addition of all-*trans*-RA caused a decrease in the number of cells over the course of the experiment, and that there was no induction of rhodopsin, Nrl, ß III tubulin, S-opsin or Crx expression. Attempts to perform RT-PCR analysis to determine whether rhodopsin was present at the mRNA level were abandoned due to the limited number of cells.

In order to sustain a greater number of cells for analysis, the cell lines were cultured in the presence of 3% charcoal/dextran treated FBS and the same concentrations of all-*trans*-RA used in the previous experiment. From the results in figures 6.5 and 6.6, the number of cells dramatically increased with the addition of FBS to the culture medium, enabling the experiment to be run for 7 days. Furthermore, and in contrast to the previous experiment, cells showed some putative neurite outgrowth as indicated by the arrows in figure 6.5. When immunocytochemical analysis of the GuRt05 (P22) cell line was performed, the cells were positive for nestin, NF200, NF160, Ki67, rhodopsin kinase and recoverin.

Strikingly, the cells also expressed ß III tubulin, a marker for immature neurons and ganglion cells, but because the DMSO control was also positive for this protein it appeared that the FBS and not the all-*trans*-RA was responsible for this up-regulation (see Chapter 5). Even with a prolonged incubation with all-*trans*-RA the cells did not express later neuronal markers e.g. S-Opsin, Crx, Nrl and most importantly rhodopsin. Further analysis of Nrl, Crx and rhodopsin by RT-PCR (figure 6.7) also failed to reveal the presence of corresponding mRNAs. The results in figure 6.6 are representative of both cell lines.

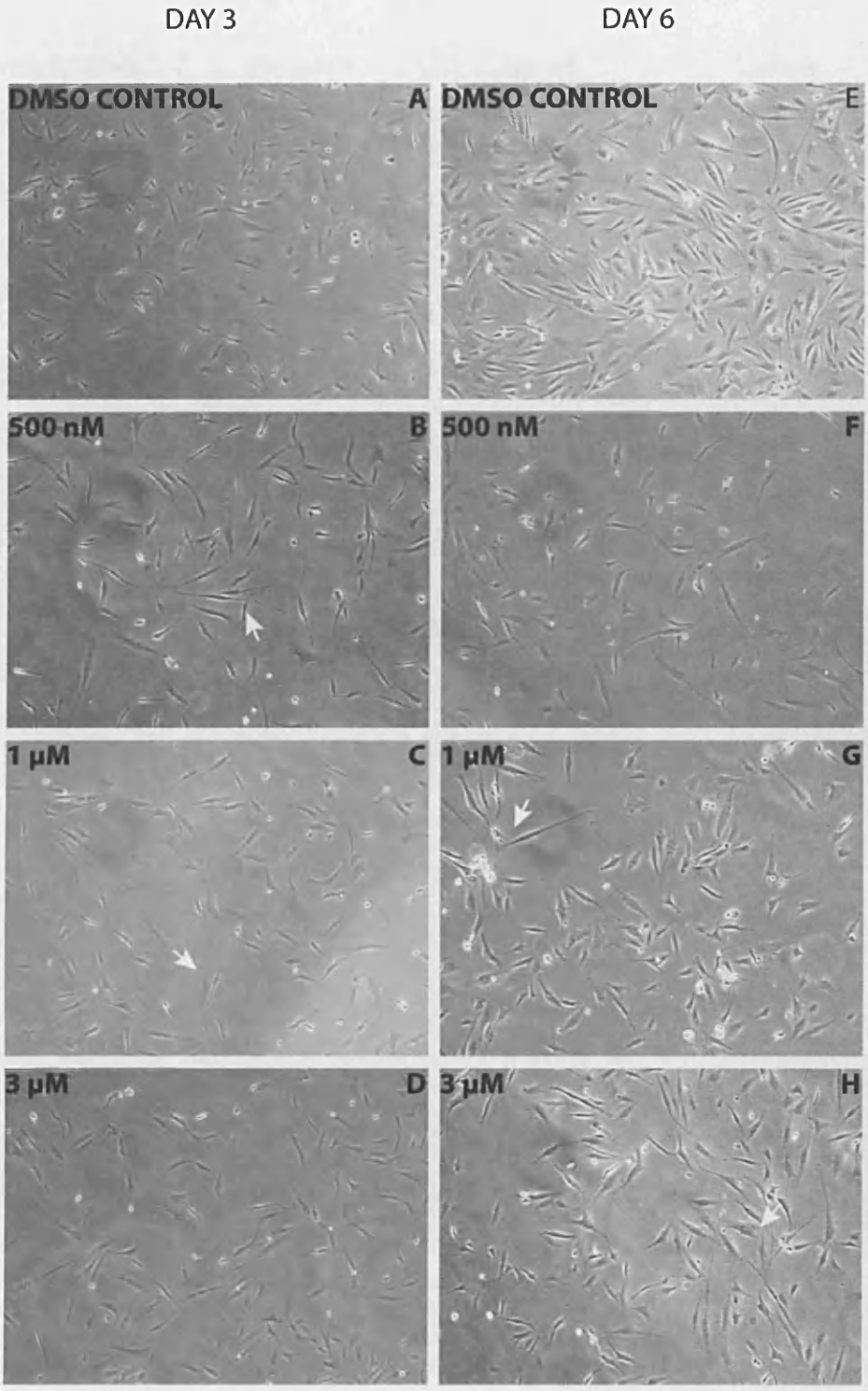

Figure 6.5 Phase Contrast images of GuRt05 cells treated with all-*trans*-RA and 3% charcoal/dextran FBS.

GuRt05 (P22) cells were cultured in the presence of 0.5-3 µM all-trans RA and 3% charcoal/dextran treated FBS for a duration of 7 days. Phase contrast images were taken at each concentration after 3 days (A-D) or 6 days of treatment (E-H). Note the appearance of cells with neurite-like processes (arrows).

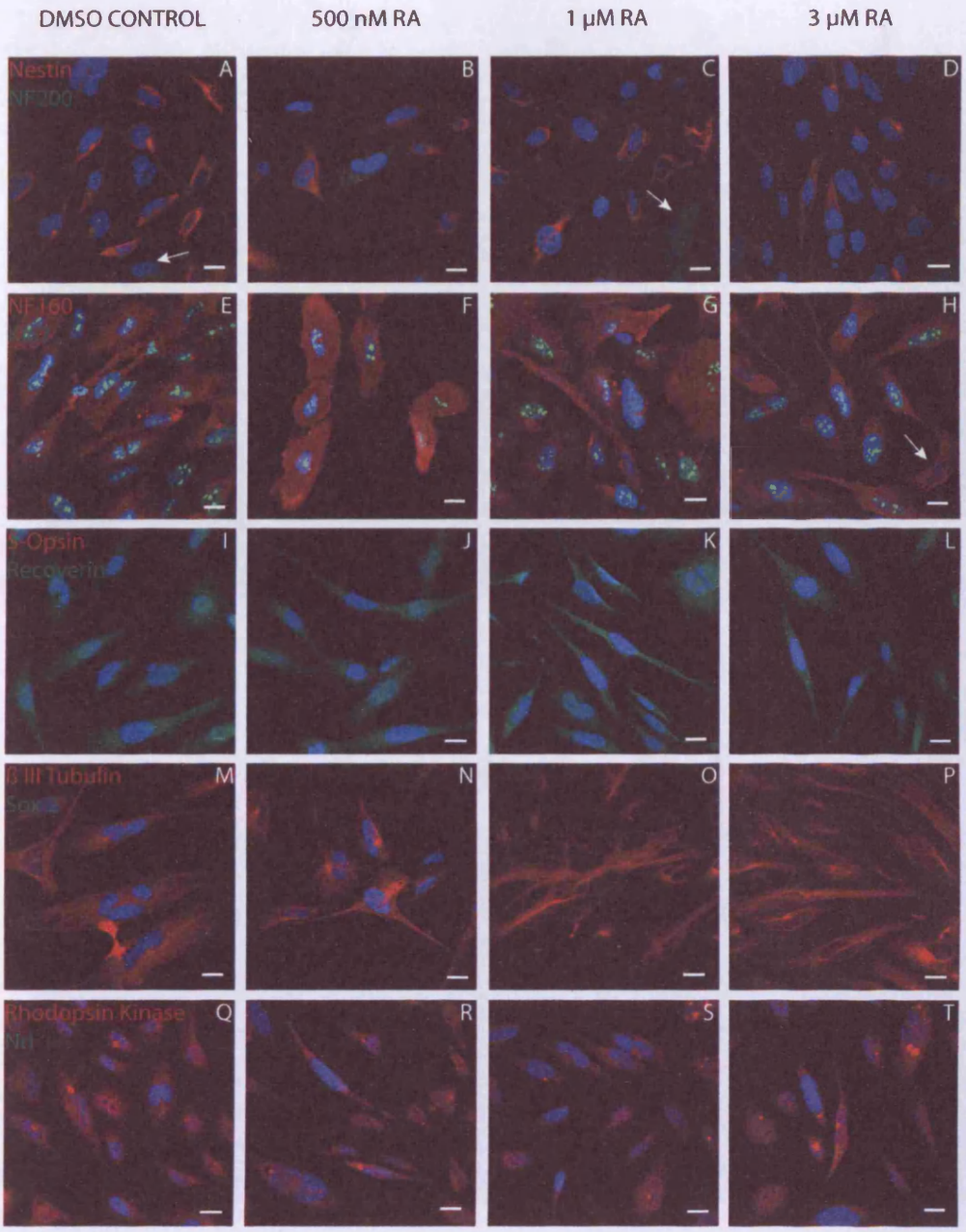

Figure 6.6 Immunocytochemical characterisation of GuRt05 cell line treated with different concentrations of all-*trans*-RA in the presence of 3% charcoal/dextran treated FBS for 7 days *in vitro*
GuRt05 (P22) cells were treated with different concentrations of all-*trans*-RA and 3% charcoal/dextran-treated FBS. (A-D) Cells continue to express the neuroectodermal stem cell marker, nestin. Note that all treated cells had a minority of NF200 positive cells (as identified by the arrows). (E-H) all of the cells immunolabelled for NF160 and were actively in the cell cycle as indicated by the positive expression of Ki67 antigen. (I-L) All cells remained recoverin positive but none were immunoreactive for the cone photoreceptor marker, S-opsin. (M-P) ß III tubulin was present in these cell lines but absent from the cells treated with all-*trans*-RA alone. There was no expression of Sox2 transcription factor. (Q-T) Note that there was no expression of Nrl which is expected to be up-regulated by all-*trans*-RA treatment. All cells at all concentrations expressed rhodopsin kinase. Cells were negative for rhodopsin and Crx (data not shown). Scale bar= 20µm.

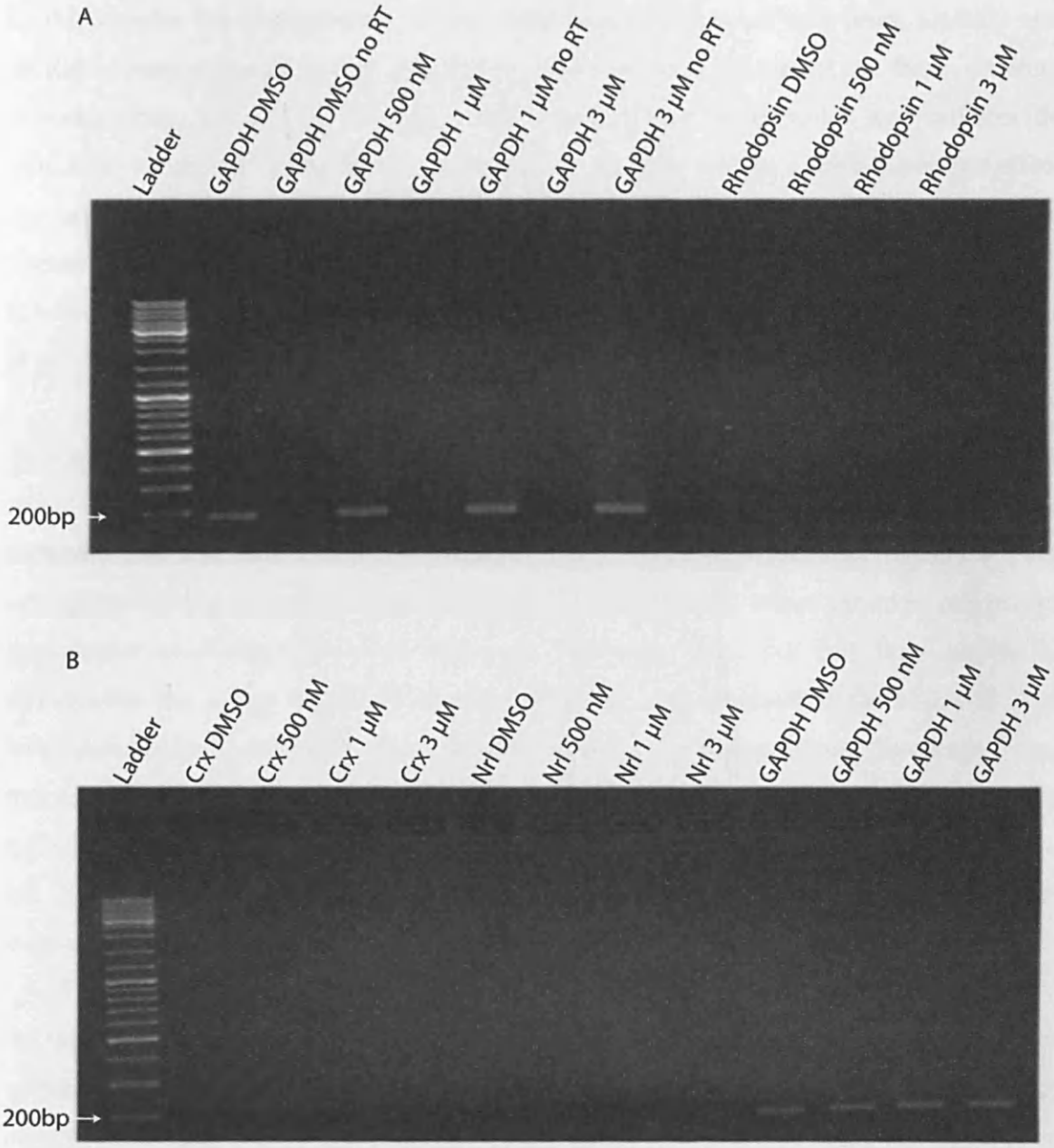

Figure 6.7 RT-PCR analysis of rhodopsin, Nrl and Crx in GuRt09 cells treated with all-*trans*-RA in the presence of 3% charcoal/dextran treated FBS
GuRt09 (P28) cells were treated for 7 days with different concentrations of all-*trans*-RA in the presence of 3% charcoal/dextran FBS. RNA was extracted from the cells and cDNA was synthesised in order to evaluate the expression of (A) rhodopsin (227bp) (B) Crx (1059bp) and Nrl (212bp). GAPDH (200bp) was run as a positive control to ensure the quality of mRNA extracted and synthesized into cDNA. Rhodpsin, Crx and Nrl were not detected via RT-PCR at any concentration of all-*trans*-RA tested or in the DMSO vehicle control.

6.4 Discussion

In this chapter the immortalised human foetal retinal progenitor cell lines, GuRt09 and GuRt05, were cultured in the absence or presence of all-*trans*-RA at three different concentrations, 500 nM, 1 μM and 3 μM. Morphologic development and cell-specific immunocytochemical markers were examined. In previous studies a dose-dependent effect was observed of RA on opsin expression in retinal chick and rat re-aggregated cell cultures. The rationale behind the use of all-*trans*-RA over 9-*cis*-RA was the proven effect all-*trans*-RA had in promoting human foetal retinal cells to differentiate into photoreceptors (Kelley *et al.*, 1995).

This study shows that varying the concentrations of all-*trans*-RA had no effect on the cell lines, and that the primary objective of inducing rhodopsin expression or photoreceptor differentiation was unsuccessful. The treated cells, minus charcoal/dextran treated FBS, did not exhibit neurite outgrowth, especially at 3 μM all-*trans*-RA, where shrunken cell bodies reminiscent of dying cells were observed. Not only were the cell lines unable to differentiate but a high degree of apparent apoptosis was observed in the both cell lines when cultured in all-*trans*-RA alone. Studies carried out in human dermal fibroblasts found that retinoids administered at the far higher concentration of 20 μM caused cell damage in a time dependent manner, with maximal apoptosis observed between 24-48 hours (Gimeno *et al.*, 2004). In comparison, the concentrations of all-*trans*-RA used in these experiments were considerably lower but still induced a similar effect.

To remedy the problem of excessive cell death, the experimental protocol was adjusted by culturing the cells in all-*trans*-RA with 3% charcoal/dextran treated FBS, which allows the cells to be treated with all-*trans*-RA for longer periods. A previous study has shown that the addition of FBS to medium results in a slower rate of RA uptake by cells (Klaassen *et al.*, 1999), it is possible that this may increase cell survival. Unlike previous studies where RA treatment was shown to induce rhodopsin expression after 1-3 days (Stenkamp *et al.*, 1993; Kelley *et al.*, 1994), there was no visible difference in the expression patterns of rhodopsin observed after RA treatment in either cell line after 7 days. All cells, including the DMSO-treated cells expressed nestin, NF160, NF200 and rhodopsin kinase. Furthermore, RT-PCR analysis failed to show the presence of Crx, Nrl or rhodopsin mRNA being transcribed by the treated cells. This seems to suggest that although the cells express

markers of photoreceptor precursors (recoverin and rhodopsin kinase), treatment with all-*trans*-RA is not sufficient to induce differentiation towards a photoreceptor lineage.

Retinoids help regulate cell proliferation in the nervous system by balancing the degree of mitosis and apoptosis that occurs within neural tissues (Söderpalm *et al.*, 2000). By controlling cell proliferation, RA regulates the transfer of cells from a proliferative to a differentiative state, but this change is highly context-specific ((Dräger and McCaffery, 1997). Moreover, RA can only have an effect on differentiation when the cells are postmitotic (Wallace and Jensen, 1999). Due to the prevalence of Ki67 expression in the cell lines at the non-permissive temperature of 37°C, this could provide an explantation as to why the cells are unable to respond to the exogenous all-*trans*-RA, and in turn, why RA was shown to have no effect on either cell line. In conclusion, even with prolonged treatment with all-*trans*-RA there may be no visible effect unless the cells are able to exit the cell cycle and become post-mitotic, whereupon all-*trans*-RA may then induce the transcription factors required for rod photoreceptor determination.

Chapter 7

The effects of ARPE19 conditioned medium on the immortalised retinal progenitor cells

Chapter 7

The effects of ARPE19 conditioned medium on the immortalised retinal progenitor cells

7.1 Introduction

Several studies have demonstrated the ability of retinal progenitor cells to respond to environmental cues and undergo cell type determination and differentiation (Levine *et al.*, 2000). Some of the first evidence of the multipotentiality of such progenitors came from observations that a diffusible factor from cultures of postnatal day one retinal cells in the mouse increases the probability that embryonic day 15 progenitors will give rise to rod photoreceptors (Watanabe and Raff 1992). The notion of neighbouring cells releasing factors that could induce differentiation has been shown to be true in the case of both retinal pigment epithelium and photoreceptors. Several studies have shown that the retinal pigment epithelium (RPE) is the source of putative diffusible factor(s) that effect photoreceptor survival and differentiation (Gaur *et al.*, 1992). This is not entirely surprising given the close association between the RPE and the photoreceptors. The RPE is a highly specialised monolayer of cells, derived from the same sheet of neuroepithelial tissue as the neural retina during development (Chen *et al.*, 2003). The RPE develops prior to other retinal cells, which would be expected if it is to play a role in the development of successive retinal cell types. Due to its location adjacent to the developing neural retina, which is destined to become photoreceptor cells, it would be highly probable that RPE would influence photoreceptor cell differentiation (Sheedlo and Turner, 1996a). Not only are these two distinct cell types adjacent and physically bound to one another, but there is a high level of interdependence between them. The RPE provides critical functions for the rod and cone photoreceptors, including outer segment phagocytosis and visual pigment regeneration. These developmental observations provide the rationale for the investigation of diffusible factors that stimulate photoreceptor differentiation *in vivo*. In this chapter, the focus is on the effect of RPE conditioned medium (CM) on the differentiation, proliferation and survival of the immortalised human foetal retinal progenitor cell lines.

7.1.1 The retinal pigment epithelium and development of the neural retina

Expression of a toxin-encoding gene in the RPE of transgenic mice resulted in the ablation of RPE cells either prior to or during their differentiation. This ablation caused the disorganisation of the retinal layers, resulting in either anophthalmia (absent eye and

complete degeneration of the pre-existing ocular structures) or microphthalmia (small eye). Interestingly, in some mice with microphthalmic eyes, the ablation was incomplete and some pigmented RPE cells survived. Directly adjacent to these RPE-rich areas the laminar structure of the retina was preserved but was devoid elsewhere. In all embryos analysed the inner layer of the optic cup was the most severely compromised by RPE ablation. This correlation between the absence of RPE cells and abnormal neural retina structure, specifically affecting the rod outer segments, indicates a major role of the RPE in the morphogenesis of the developing retina (Raymond and Jackson, 1995). This essential role of the RPE in the maintenance of the neural retina is highlighted further in the animal model of the Royal College of Surgeon (RCS) rat. In this rat, there is a defect in the RPE cells which has been proven to cause the subsequent loss of photoreceptors (Mullen and LaVail, 1976). All these examples point to the vital role of the RPE in the development of the neural retina, and in particular photoreceptors. This raises the question, can the RPE also contribute to the survival and differentiation of these cell types by the secretion of diffusible factors?

7.1.2 Neuronal differentiation induced by RPE conditioned medium

It has been known for many years that the RPE synthesizes and secretes proteins (Bryan and Campochiaro, 1986) such as platelet derived growth factor (PDGF) (Campochiaro *et al.*, 1989), glycosaminoglycans (Stramm, 1987), and basic fibroblast growth factor (bFGF) (Bost *et al.*, 1992) amongst many others. However, the factors responsible for the inductive effects in the development of the neural retina, in particular the photoreceptor cells, had not been identified. Work carried out by Tombran-Tink and Johnson (1989) found that human foetal RPE-cell conditioned medium caused Y79 human retinoblastoma cells to express neuronal characteristics such as long neurite-like processes and to express neuronal markers, such as NF200 and neuron-specific enolase (a marker for mature neurons). For the first time this was clear evidence that RPE cells could induce neuronal differentiation via the factors secreted into the culture medium. This led to several other groups observing similar results on neuronal development in the presence of RPE-conditioned medium. In these studies, it was demonstrated that RPE conditioned medium, and more specifically the factor(s) secreted into the medium, caused an increased number of photoreceptor cells in the developing chick embryonic neural retina (Spoerri *et al.*, 1988).

Gaur and colleagues (1992), found that photoreceptor differentiation (confirmed with opsin positive cells) and survival were induced, this latter observation being reinforced by experiments that confirmed that RPE cells had the ability to stimulate retinal survival and increased cell proliferation rates (Liu *et al.*, 1988). Most of these studies used freshly harvested RPE cells from either embryonic, foetal or early post-natal tissue. However, studies using spontaneously transformed neonatal rat RPE cell lines, and the conditioned medium from these cells also proved to have similar effects with the promotion of proliferation, neurite outgrowth and the expression of photoreceptor cell-specific markers (Sheedlo and Turner, 1996b).

7.2 Experimental design and objectives

ARPE19 cells were cultured in their normal medium of DMEM:F12 media plus L-GlutaMAX™ supplemented with 10% (v/v) Foetal Bovine Serum (FBS) and 1.0% (v/v) Penicillin/Streptomycin for 1 week until confluent. The cells were then washed thoroughly with PBS and cultured in serum-free medium (DMEM:Fl2 and 1.0% (v/v) Penicillin/Streptomycin for a duration of either 1, 3 or 5 weeks without medium change. This medium was then collected, centrifuged to remove any cell debris, and used as conditioned medium (CM) on the immortalised human foetal retinal progenitor cell lines. The GuRt09 and GuRt05 cell lines were seeded on to laminin-coated dishes or flasks at an initial seeding density of 1.0 x 10^4 cells/cm^2 and cultured in either 100% CM or 20% CM (80% plain DMEM:F12). As a control, cells were cultured in plain DMEM:F12 and 1.0% (v/v) Penicillin/Streptomycin that had not been conditioned prior to use. All cells were cultured in CM or control medium for 7 days and then fixed with 4% PFA for immunocytochemical analysis or flow cytometry. The rationale for leaving the medium to be conditioned by the ARPE19 cells for long periods was that extracts of certain secreted factors may increase in expression with time. Thus, extending the conditioning period enabled us to determine whether there was a temporal effect on the factors secreted by ARPE19 cells and their potency to induce differentiation.

7.2.1 ARPE19, a human retinal pigment epithelial cell line

The experiments in this chapter utilised a spontaneously arising and widely used human RPE cell line, ARPE19. The cell line was derived from the globes of a 19 year old male donor and has been extensively studied (Dunn *et al.*, 1996). The cell line has a normal karyology and although non-pigmented, retains the characteristic 'cobblestone' morphology of epithelial cells. The cells also express some of the characteristic markers of differentiated RPE cells *in vivo*. Hence it was hypothesised that these cells would provide a reasonable source of secreted factors for these experiments.

ARPE19 cells that were cultured until confluent and then used to condition DMEM:F12 medium for a duration of 1, 3 or 5 weeks survived reasonably well in the absence of FCS (figure 7.1). ARPE19 cells at the 1 week stage clearly retained their characteristic epithelial morphology. However, after 5 weeks in culture, some cells had adopted a spindle-like morphology, and although there were still living cells present they had little resemblance to the 1 week or 3 week cultures.

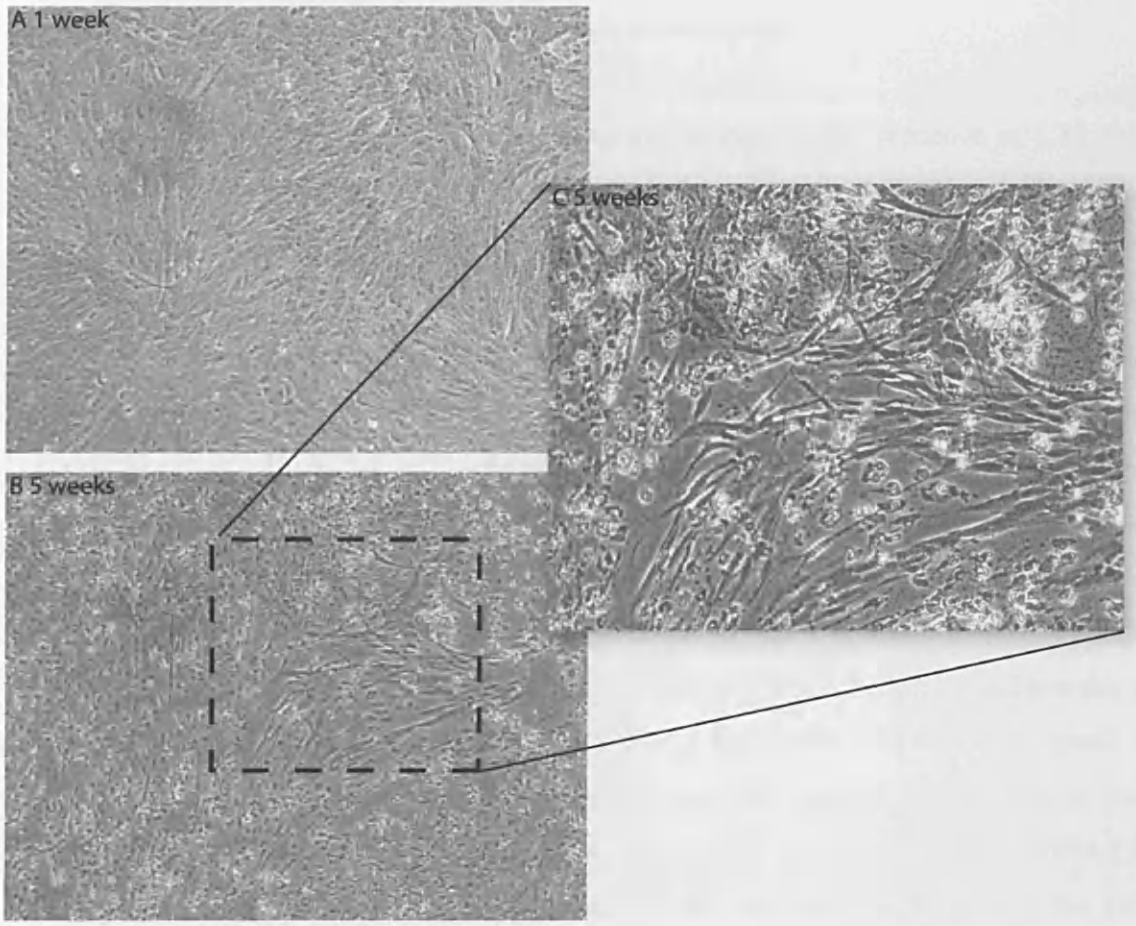

Figure 7.1 Human ARPE19 cell lines used to condition serum-free medium
Human ARPE19 cells were grown to confluence for 1 week, at which point serum-free medium was added to the cells for 1 week, 3 weeks or 5 weeks. (A) Phase image of cells after 1 week in the presence of FCS and then cultured in medium for 1 week to yield conditioned medium. (B) Image of ARPE19 cells left for 5 weeks to condition DMEM:F12 medium. There were still cells growing in the culture flask. (C) Interestingly, some cells had altered their characteristic cobblestone morphology and adopted a spindle-like appearance.

7.3 Results

First the morphology of the GuRt09 (P22) cells was examined (figure 7.2) during culture for 1, 4 and 7 days at 37°C in the presence of either DMEM:F12 alone (control), DMEM:F12 supplemented with 20% ARPE19 conditioned medium (CM) or 100% CM. The CM in this experiment was conditioned by confluent ARPE19 cells for 1 week before being used as culture medium on the human foetal retinal progenitor cells. As inter-experimental variability was minimal, results of representative experiments will be shown throughout this chapter to simplify data presentation.

Initial observations indicated that all cultures maintained in the presence of CM grew more rapidly than compared to the control. It was also apparent that at day 4 (figure 7.2, E and I) some cells were exhibiting altered morphology and becoming more elongated, with long processes, similar to the neurite outgrowth observed by Gaur *et al.*, (1992). By day 7, the majority of cells in the control wells had died. However, the cells cultured in the presence of CM were growing robustly with approximately 50% confluence reached by cells in 20% CM, whilst those cells in the 100% CM were slightly less dense. To gain further insight into the proliferation rates, cell numbers were determined under all three conditions over an eight day period. Cells were seeded at an initial density of $9.5x10^4$ per well (figure 7.3). By day 2, cells cultured in 100% CM had $3.8x10^4$ cells per well, whilst the cells in 20% CM had $2.0x10^4$ and the controls had the lowest number of cells at $1.5x10^4$. On day 4, the number of cells in the 100% CM reached a peak of $7.5x10^4$ per well, and again both the 20% CM and the control had far fewer cells ($3.0x10^4$ and $0.5x10^4$ respectively). By day 6, the number of cells cultured in 100% CM had dropped to $3.5x10^4$, which was approximately the same number of cells in the 20% CM. The number of cells in the 20% CM continued to increase over the next 2 days whilst the cells in 100% CM continued to decline. By day 8, the number of cells treated with 20% CM was just under $5.0x10^4$, almost double that of the cells treated in 100% CM. The number of cells in the control wells stayed consistent at $0.5x10^4$. Thus, there was a marked increase in cell numbers when cells were cultured in ARPE19 CM compared to the control.

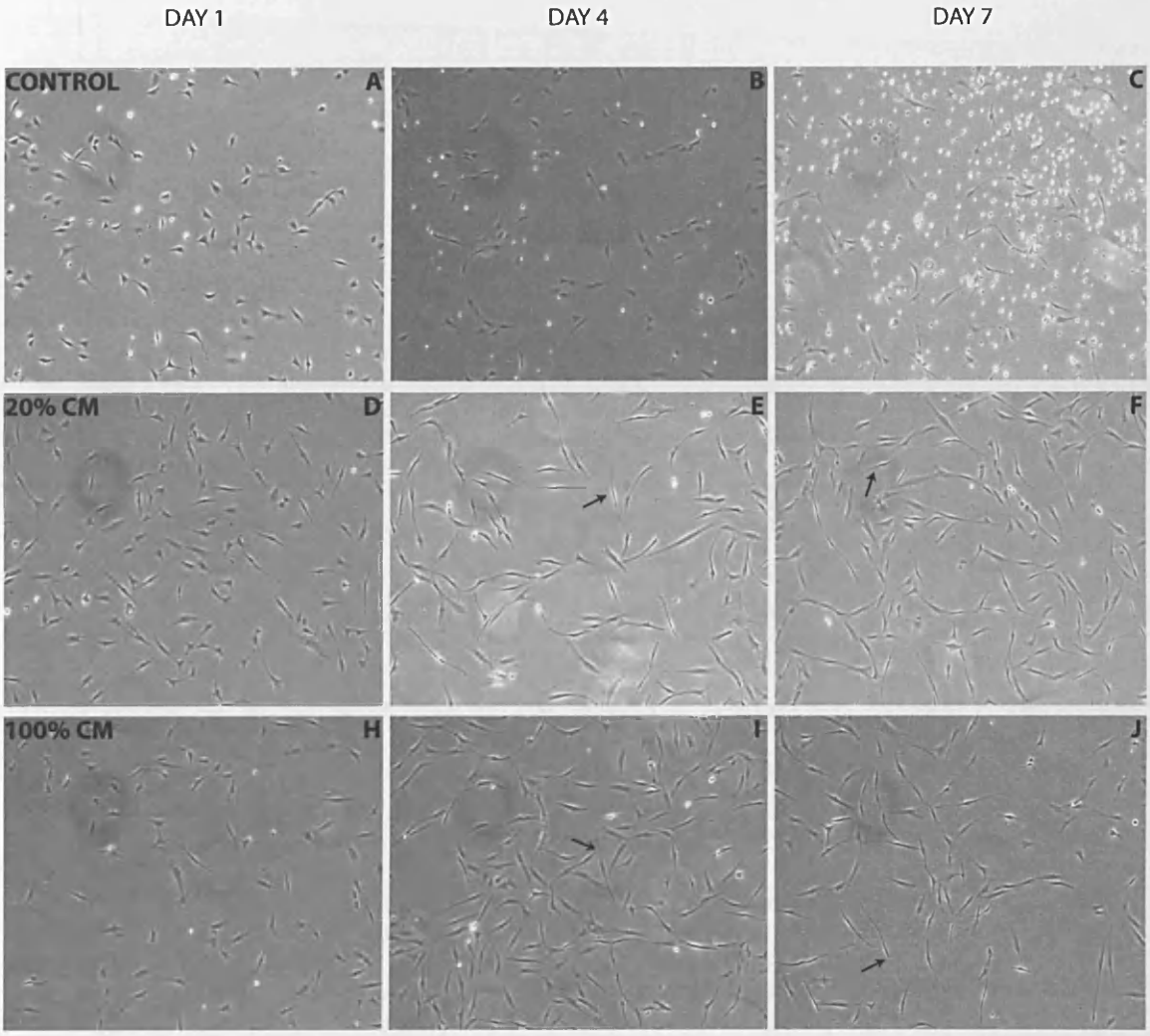

Figure 7.2 Morphological differences between ARPE19 Conditioned Medium-treated and control cells
GuRt09 (P22) cells were cultured in ARPE19 medium that had been conditioned for 1 week. (A,B,C) Control cells cultured in DMEM:F12 medium for 7 days. (D,E,F) Cells cultured in 20% CM and 80% plain medium. (H, I, J) Cells cultured in 100% CM for 7 days. Note the arrows identifying cells with long processes in (E,F,I and J). Compared to the control cells the cells cultured in CM have a high cell number possibly due to stimulation of proliferation or ehnanced cell survival due to factors with the conditioned medium.

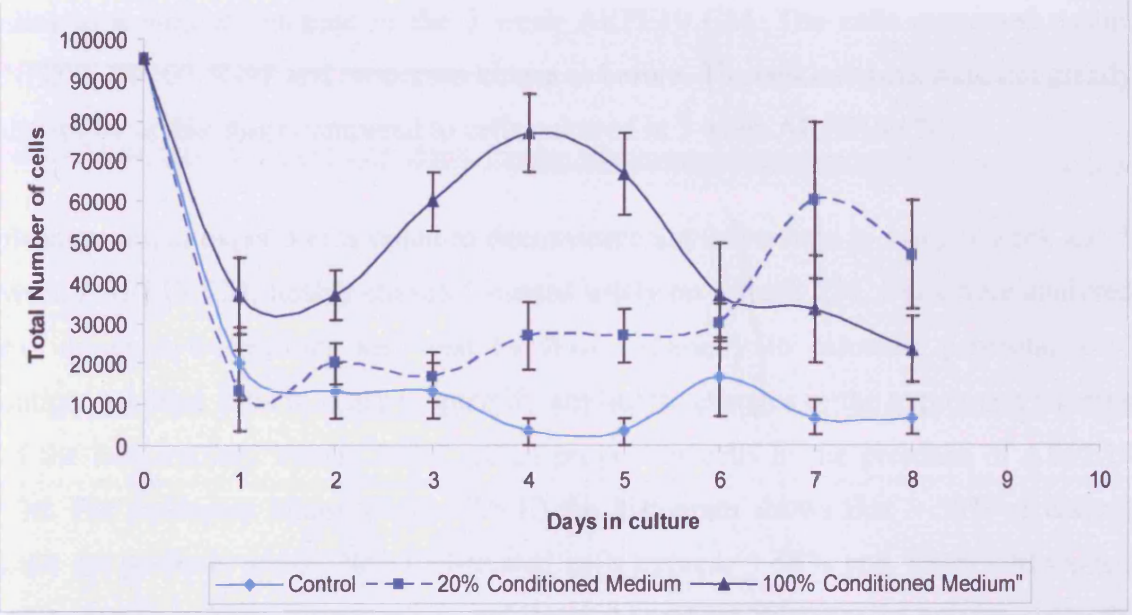

Figure 7.3 Proliferation of GuRt09 cells in the presence or absence of ARPE19 Conditioned Medium
GuRt09 (P22) cells were seeded at an initial density of 95,000 cells per well and cells were trypsinised and counted each day for total cell numbers. The cells in control medium (DMEM:F12) had the least number of cells throughout the course of the experiment. Cells in the presence of 100% and 20% ARPE19 Conditioned Medium (CM) had greater number of cells compared to the control. Cells cultured in 100% CM had the greatest increase in cell numbers at the beginning of the culture period with the highest value of approximately 75,000 cells in total at 4 days. But by day 6 the numbers had dropped to 35,000 cells. At this point the cells cultured at 20% had the highest number of cells in culture. The data are presented as the mean total number of cells $\pm$ SEM.

Immunocytochemical analysis of the progenitor cell lines cultured in 3 week conditioned medium did not show a great deal of difference. GuRt09 (P18) cells after 7 days treatment with 3 week ARPE19 CM (figure 7.4) revealed no change in protein expression when compared with cells cultured in control medium. Earlier results indicated that the addition of ARPE19 CM may increase the number of cells over the course of the experiment compared to the control. However, exposure to 3 week ARPE19 CM did not induce rhodopsin, Nrl, ß III Tubulin, S-Opsin or Crx expression, as judged by immunocytochemistry.

Results for GuRt05 cells cultured in 5 week ARPE19 conditioned medium (figure 7.5) showed a similar outcome to the 3 week ARPE19 CM. The cells expressed nestin, NF200, NF160, Ki67 and rhodopsin kinase as before. The cell numbers were not greatly dissimilar at this stage compared to cells cultured in 3 week ARPE19 CM.

Because initial experiments failed to demonstrate any advantage to using 3 week and 5 week ARPE19 CM, further studies focussed solely on 1 week CM. Cells were analysed by immunocytochemistry and also by flow cytometry to calculate percentages of antigen positive cells in order to quantify any subtle changes in the expression patterns of the immortalised human foetal retinal progenitor cells in the presence of ARPE19 CM. For rhodopsin kinase (figure 7.6 E) the histogram shows that ~ 58% of control cells are positive, whilst 20% CM-treated cells express ~ 58% and 100% CM-treated cells express ~ 72%. These values and those throughout this chapter were statistically analysed using the Student t-test, where $p=<0.05$ is significant. These results show that the expression patterns of rhodopsin kinase were not significantly altered in cells cultured with or without CM. Although not tested, it is likely that similar results would have been obtained for 3 week and 5 week ARPE19 medium, since the confocal images of rhodopsin kinase staining were not dissimilar to the 1 week ARPE19 CM.

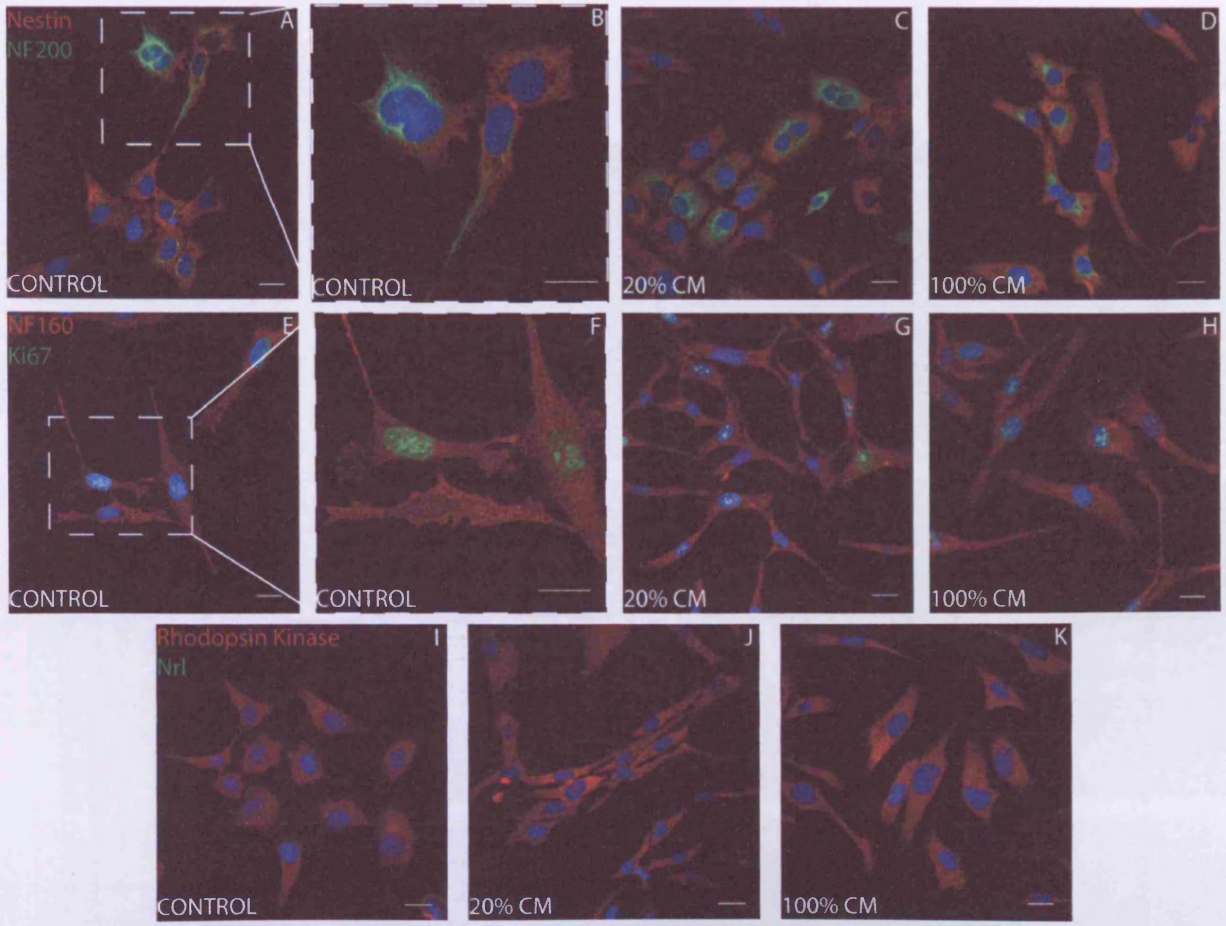

Figure 7.4 Expression profile of GuRt09 cells treated with ARPE19 (3 week) conditioned medium
GuRt09 (P18) cells were either cultured in 20% CM or 100% CM for 7 days and then fixed and stained for various markers. Cells were positive for nestin and NF200 (A-D), NF160 and Ki67 (E-H) and rhodopsin kinase (I-K). Nrl, Rhodopsin, Crx and ß III Tubulin were undetectable by immunocytochemistry. Cells in the CM-treated cultures were more numerous than in the control cultures, but there was no visible difference in expression patterns between the conditions. The boxed regions in A and E are shown at a higher magnification in B and F respectively. Scale bar= 20 µm.

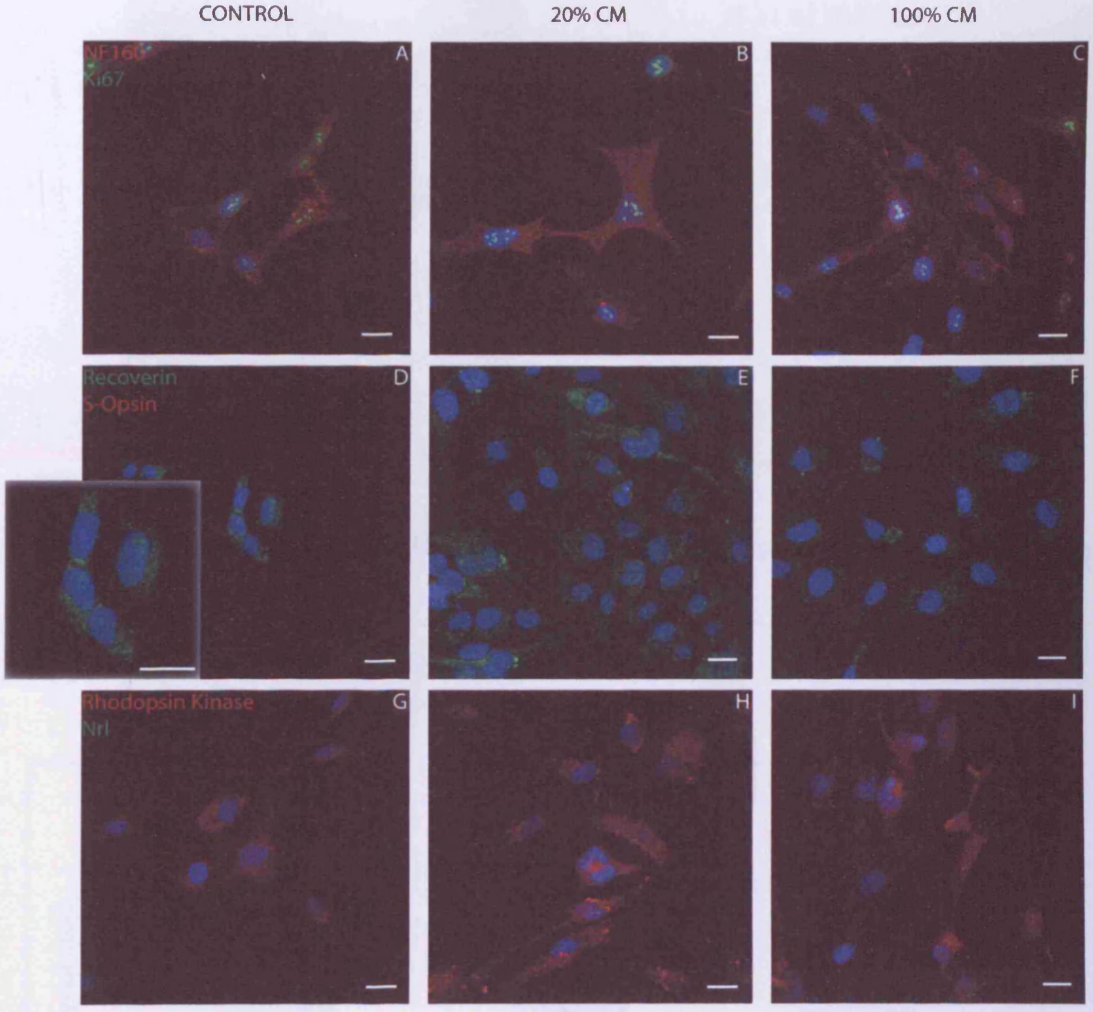

Figure 7.5 Expression profile of GuRt05 cultured in 5 week ARPE19 Conditioned Medium
GuRt05 cells (P26-P28), were cultured in conditioned medium derived from ARPE19 cells grown for 5 weeks prior to removal of medium. The cells were grown in three different conditions (A,D,G) DMEM:F12 medium (which was the control for this experiment), (B,E,H) DMEM:F12 medium supplemented with 20% (5 week) ARPE19 CM and (C, F, I) cells were grown in 100% (5 week) ARPE19 CM. Rhodopsin, Sox2, and ß III Tubulin and Crx were also examined but these antigens were undetectable. There was no significant difference between the expression patterns of the cells under these different conditions. However, the cells grown in the conditioned medium did show an increase in cell numbers compared to the control. Scale bar=20 µm.

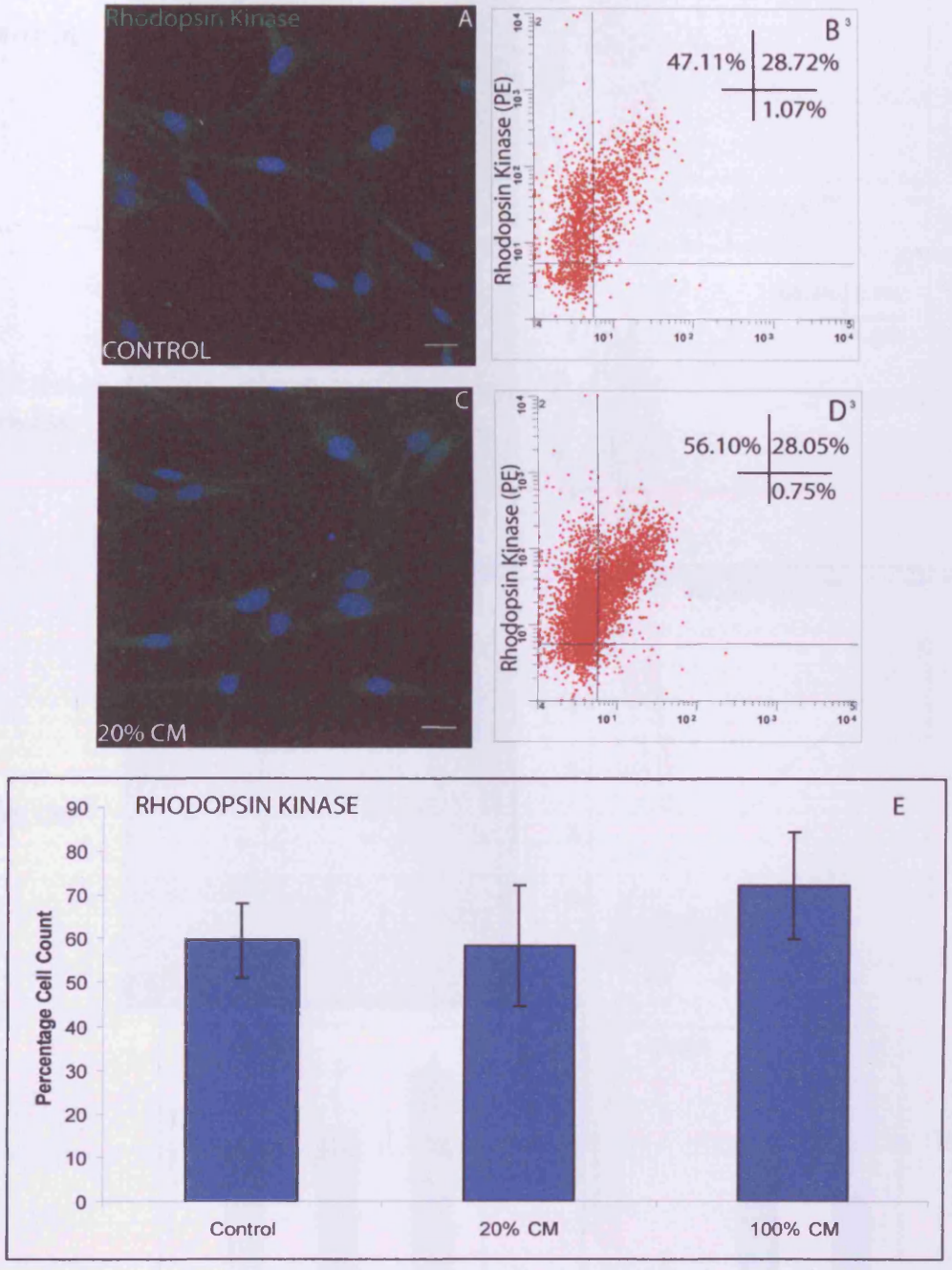

Figure 7.6 Rhodopsin kinase expression in retinal progenitor cells cultured in ARPE19 CM
(A,C) GuRt09 (P22) cells were cultured in 1 week ARPE19 conditioned medium and then fixed either for confocal imaging or flow cytometry analysis, scale bar= 20 μm. (B, D) Dot plots representing the percentage value for rhodopsin kinase expression for cells in control medium (B) and in 20% CM (D). The results from the dot plots were coverted into a histogram (E) displaying total percentage as means ± SEM (n=3). As the confocal images show there is no significant difference betweenthe levels of rhodopsin kinase expression of cells cultured in the presence or absence of ARPE19 CM.

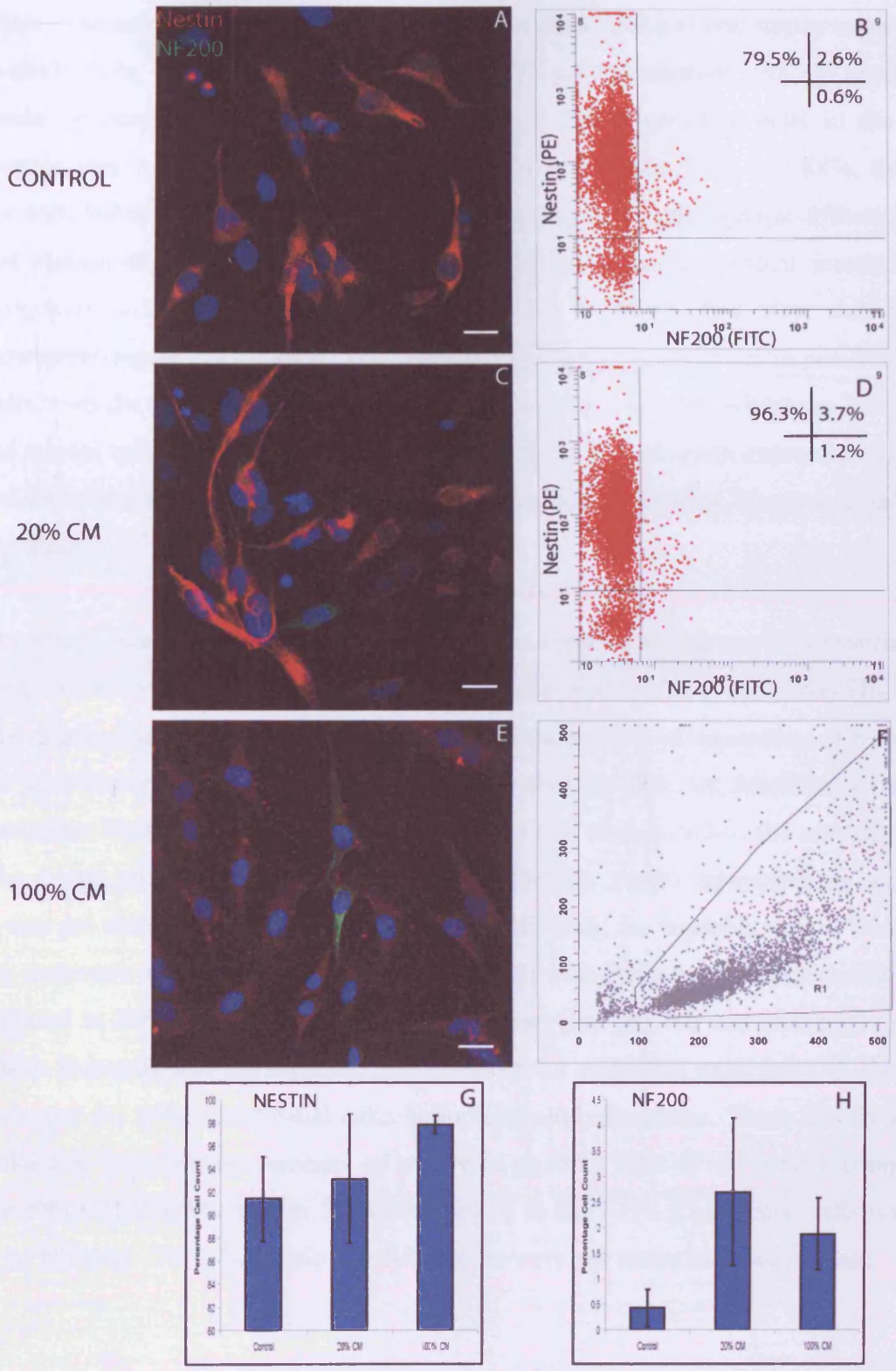

Figure 7.7 Expression of nestin and NF200 in the presence and absence of ARPE19 Conditioned Medium

(A,C, E) Confocal images of GuRt09 cells cultured in 1 week ARPE19 conditioned medium at varying percentages (control, 20% or 100%). Cells are positive for both nestin and NF200, scale bar = 20μm. (B,D) Flow cytometry analysis to measure the percentage of nestin and NF200 positive cells in the control and 20% CM. (F) An image of the total population analysed by the flow cytometer, the drawn quadrant indicates the gated population which excludes cell debris and the majority of dead cells. (G, H) Flow cytometry data were collected and represented in the form of histograms, data presented as total percentage mean ± SEM (n=3).

Flow cytometry analysis was then used to investigate NF200 and nestin expression in GuRt09 cells. The results for NF200 (figure 7.7) were consistent with the observations made by confocal microscopy. The number of NF200 positive cells in the control sample was 0.43% (figure 7.7 H), substantially lower than 2.7% or 1.88%, the values for cells cultured in 20% CM and 100% CM respectively, although the differences were not statistically significant. Robust nestin expression was a constant feature of both progenitor cell lines, regardless of the medium in which they were cultured. The histogram (figure 7.7 G) appears to indicate a modest increase in nestin positive GuRt09 cells, with the control cells expressing the lowest level of nestin, whilst the 20% CM has the medial value and the 100% CM had the highest level of nestin expression. However, the difference of 6.44% between the control cells and the 100% CM was not statistically significant.

In order to examine photoreceptor markers, the expression patterns of recoverin and S-opsin were investigated either by immunocytochemistry or flow cytometry (figure 7.8). The confocal images (figure 7.8 A-C) show a similar level of expression of recoverin in all three experimental conditions. The flow cytometry data are presented as a single-parameter histogram for recoverin (figure 7.8 D-F) as opposed to the normal dot plot. The x-axis represents fluorescence intensity and the y-axis represents the number of events per channel. This display is ideal for comparing the negative control (black) with the recoverin expression in control cells (red), and the recoverin expression in cells cultured in 20% CM (blue). Figure 7.8 F shows that the red and blue peaks overlap, which indicates that the fluorescence intensity for recoverin expression in the control cells and the 20% CM treated cells is approximately the same. There is only a 6.33% difference between the numbers of recoverin positive cells in the control compared to the 20% CM treated cultures. Recoverin levels in the 100% CM-treated cells was found to be lower at ~73%, but again the differences were not statistically significant.

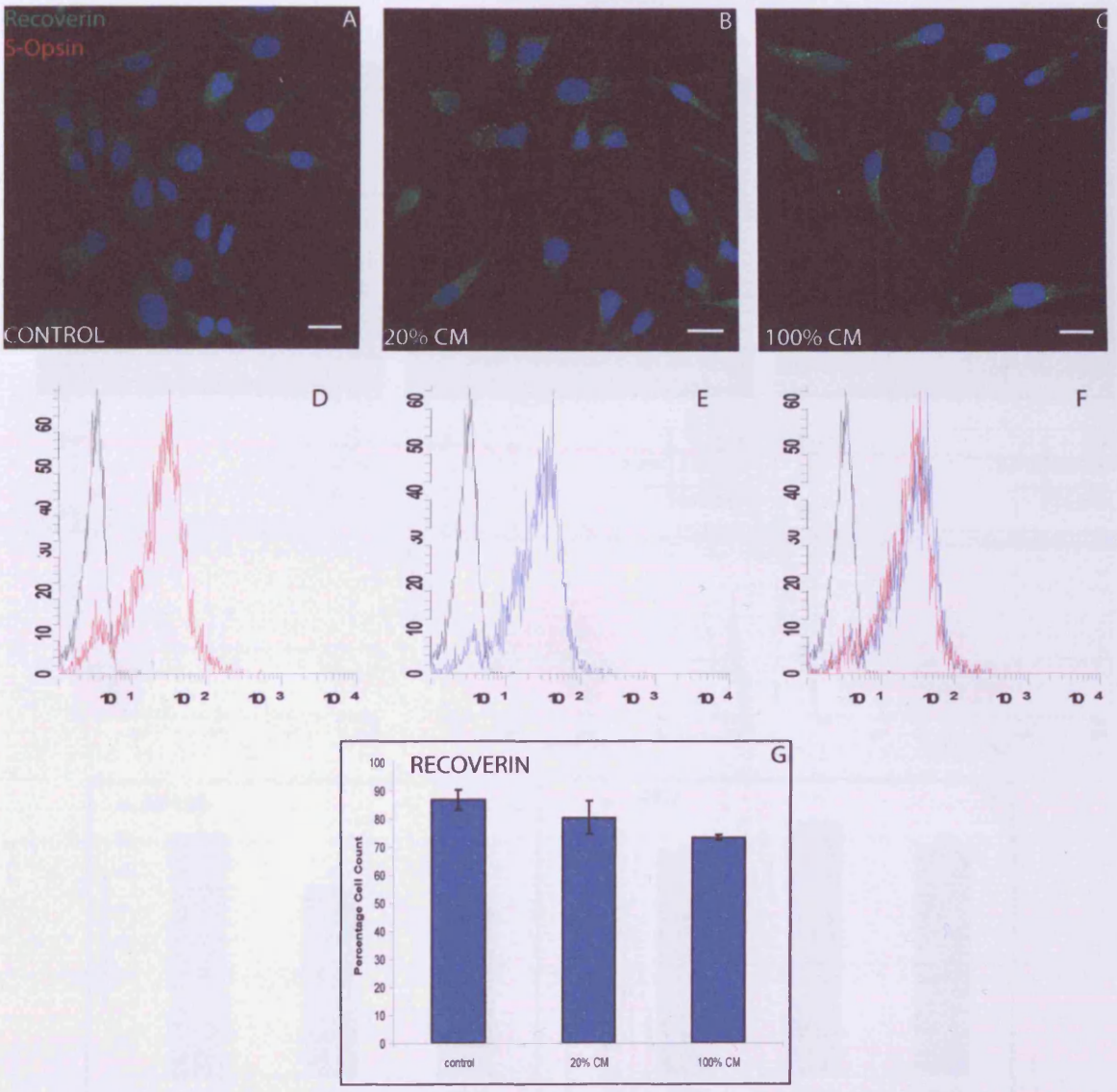

Figure 7.8 Recoverin expression in GuRt09 cells treated with ARPE19 Conditioned Medium
(A, B, C) Representative confocal images of cells grown in 1 week ARPE19 conditioned medium (CM) for 7 days and then fixed and stained for recoverin and S-Opsin. S-Opsin was undetectable in all conditions, scale bar= 20μm. (D,E, F) Flow cytometry analysis was also carried out on these cells and S-Opsin was undetected here also. Representative histograms overlays in (D) represents cells cultured in control medium stained for recoverin (red) or negative control (black). (E) The blue peak is representative of the data for cells cultured in 20% CM and then stained for recoverin, the black peak represents the negative control. (F) Merging both the peaks of control and 20% CM shows no great difference in mean fluorescence intensity (MFI). (G) Percentages obtained from flow cytometry were represented in the form of a histogram showing the difference in recoverin expression across the 3 conditions. Data is represented as total percentage mean ± SEM (n=3).

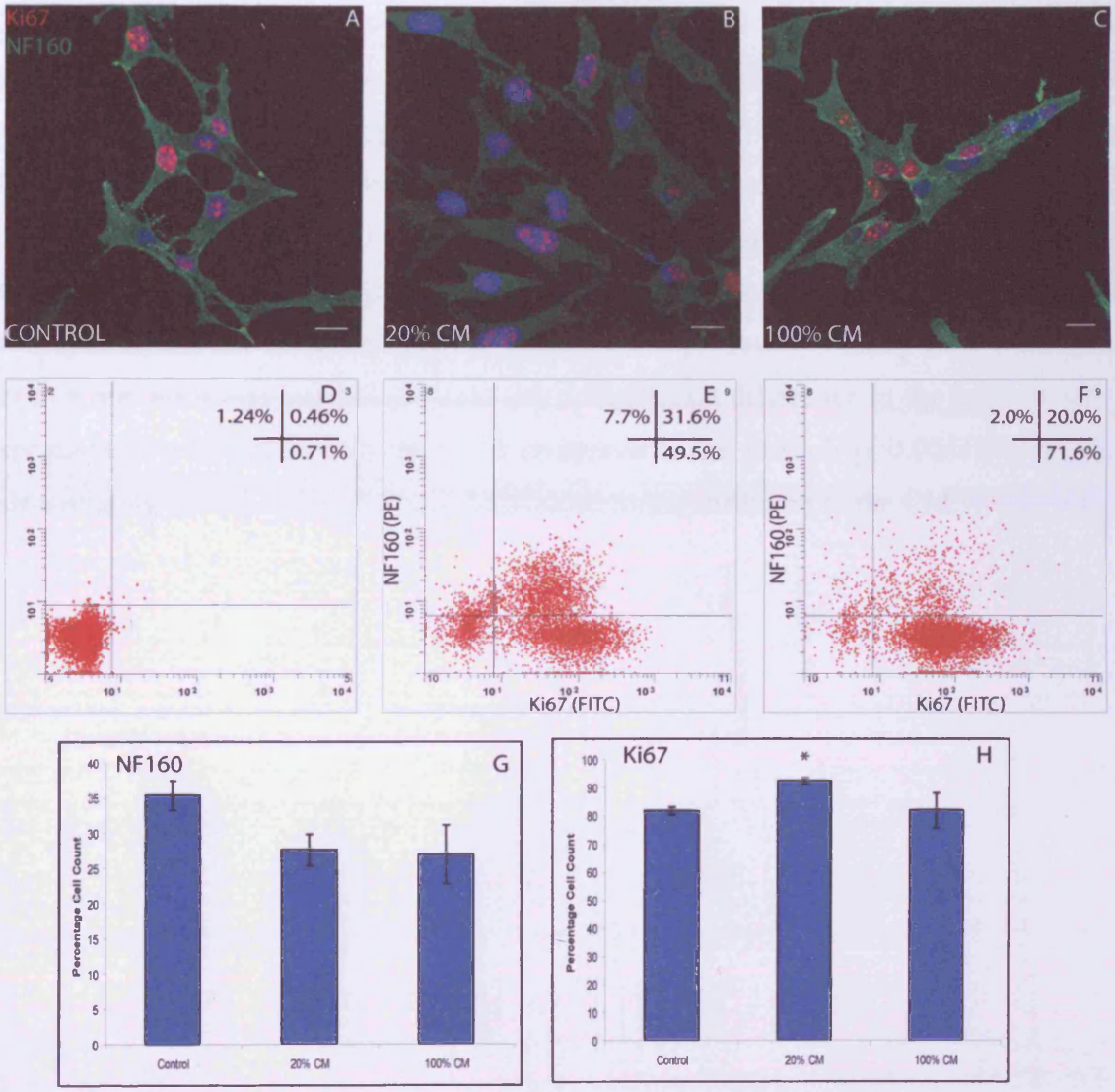

Figure 7.9 Expression patterns and flow cytometry analysis of NF160 and Ki67 in GuRt09 cells cultured in 1 week ARPE19 conditioned medium

GuRt09 (P22) cells were cultured in 20% or 100% CM for 1 week at which point they were stained for confocal analysis or flow cytometry analysis. (A,B,C) Representative images of cells stained for Ki67 and NF160, scale bar= 20 µm.(D, E, F) Dot plots representating, from flow cytometry analysis, a negative control (D), control cells stained for Ki67 and NF160 (E) and cellscultured in 20% CM (F). (G, H) Histograms with all the data collected from the flow cytometry analysis for NF160 expression (G) and Ki67 (H). All values are presented as total mean percentages ± SEM (n=3).

The expression patterns of NF160 and Ki67 were also analysed (figure 7.9 A-C) and there did not appear to be any apparent difference between the culture conditions as viewed from the confocal images. During flow cytometry analysis, negative control samples were always tested in conjunction with the experimental samples (figure 7.9 D) to detect the presence of false positives. Analysis of NF160 expression revealed that ~35% of cells in the control culture were positive for NF160 (figure 7.9 G), compared to ~27% in the 20% CM and ~26% for the 100% CM. There was no apparent difference in NF160 expression between the three conditions.

Despite the absence of any changes in gene expression in cells grown in CM, figure 7.2 shows increased numbers of cells in the ARPE19-CM treated sample compared to the control. This suggests that factors within the CM have a stimulating effect on proliferation, but not on differentiation. On the basis of flow cytometry analysis (figure 7.9 E, F and H) it was found that there was a significant difference in the level of Ki67 expression in cells cultured in 20% CM compared to the control ($p=0.004121$), further substantiating the existence of a proliferation stimulus attributed to the CM.

7.4 Discussion

In this chapter, the immortalised human foetal retinal progenitor cell lines, GuRt09 and GuRt05 were cultured in the absence or presence of ARPE19 conditioned medium. Morphologic development, cell growth rates and immunocytochemical markers were examined visually and quantitatively.

The results here show that varying the time the medium was conditioned with ARPE19 cells had little to no effect on the cell lines, and that regardless of the conditions there was no induction of rhodopsin or apparent differentiation towards a photoreceptor lineage. The major discernable effect of cultures in CM was that a significant number of cells were mitotically active in 20% CM compared to the control. A similar effect on proliferation has been noted by Sheedlo and Turner (1996b). A possible candidate factor known to be secreted from the RPE and which has been shown to stimulate growth rates in these cells is bFGF (Bost et al., 1992), which could be acting alone or in combination with other factors. As has been shown in Chapter 4, bFGF can indeed increase Ki67 expression in these cell lines.

However, the increase in Ki67 expression and cell proliferation could be one of the reasons differentiation does not occur. For a cell to differentiate, it must exit the cell cycle and commit to a particular lineage. Another diffusible factor identified in RPE conditioned medium, and known to have a potent effect on neuronal differentiation, is Pigment Epithelium-Derived Factor (PEDF) (Tombran-Tink and Johnson, 1991). At a concentration of 1 nM, PEDF has been shown to switch off proliferation in Y79 cells and cause neurite extensions and increased expression of antigens associated with differentiated neurons (Tombran-Tink and Barnstable, 2003). These observations exemplify the importance of terminating proliferation in order for differentiation to occur.

The age and source of the RPE cells could also have an effect on the properties of the CM as younger cells may deliver a more potent cocktail of diffusible factors. Since ARPE19 cells are immortalised and only have a partial RPE phenotype, their ability to secrete certain key factors may be compromised. However, transformed cells have been used in previous studies and positive effects have been observed (Sheedlo and Turner 1996b). Some papers have commented on the need to co-culture the RPE with the progenitor cells to obtain differentiation (Liu *et al.*, 1988). Perhaps the diffusible factors

only function over a short distance and/or have a short functional half-life. In conclusion, these experiments demonstrate that ARPE19 CM has a significant effect on cell proliferation but has no effect on the differentiation of these immortalised human foetal retinal progenitor cell lines. The enhanced proliferation may prevent the cells from becoming post-mitotic and differentiating.

Chapter 8

Transplantation of human foetal retinal progenitor cells into the diseased and developing in vivo environments

Chapter 8

Transplantation of human foetal retinal progenitor cells into diseased and developing *in vivo* environments

8.1 Introduction

In previous chapters the aims were to elucidate the potential of immortalised human foetal progenitor cells to differentiate towards retinal specific cell types. The ultimate goal of this research has always been to demonstrate proof of principle that immortalised progenitor cells such as these might be used in a clinical setting to treat degenerative eye diseases by retinal cell transplantation. Restoring and replacing lost photoreceptors may alleviate disease and maintain some degree of functional vision in patients who would otherwise be blind. The most rigorous assay of a cell's ability to develop and differentiate is to observe cell behaviour in an *in vivo* environment. This chapter assesses the effects of transplanting the unimmortalised human foetal retinal progenitor cells, GS076, and the immortalised human foetal retinal progenitor cell line, GuRt09, into immunosuppressed neonatal Lister, non-dystrophic and dystrophic RCS rats. Observations were made regarding the survival and integration of these cells in the host retinal environment.

Investigations into the ability of transplanted cells to restore visual components have been going on for decades. In 1986, Turner and Blair created lesions in rat retinae and grafted neonatal retinal tissue into these sites, observing the survival and possible integration of tissue into the host neural structure. This established a paradigm for what could be achieved using this approach, and raised the possibility that with graft integration there was a possibility that appropriate connectivity between graft and host may be made to relay proper visual signals and thus restore sight in diseased animal models.

8.1.1 The Royal College of Surgeons rat

Several animal models have been used to experimentally test whether retinal transplants can preserve a damaged retina. In this chapter the widely studied and well established Royal College of Surgeons (RCS) rat was employed. This animal model has a recessive inherited retinal degeneration that leads to a defect in the retinal pigment epithelium (RPE), resulting in an abnormally low rate of rod outer segment phagocytosis (Litchfield *et al.*, 1997). The accumulation of outer segment debris leads to the

progressive postnatal loss of photoreceptor cells. With the use of positional cloning, the defect was discovered to be a deletion of 409 bases in the 5' sequence of the gene encoding the Mer receptor tyrosine kinase (D'Cruz *et al.*, 2000). During the first 3 months the majority of photoreceptors degenerate (Dowling and Sidman, 1962), and due to certain similarities between the RCS rat and the degenerative diseases of age-related macular degeneration (AMD) and Retinitis Pigmentosa (RP), it has become a much used animal model for transplantation studies.

8.1.2 RPE transplantation and the RCS rat

Many attempts have been made to delay or arrest the degeneration of photoreceptors in the RCS rat. Several studies have shown that grafted RPE cells can slow degeneration and rescue some photoreceptor functionality (Little *et al.*, 1996; Gouras *et al.*, 1989). The use of a SV40 immortalised RPE cell line has also been demonstrated to have functional efficacy when transplanted into the subretinal space of RCS rats (Lund *et al.*, 2001). The potential to promote cell survival is not confined to RPE grafts alone, other cell types such as immortalised brain-derived cells have also been shown to promote in photoreceptor survival (Wojciechowski *et al.*, 2002).

Administering growth factors either via cells genetically engineered to stably over-express certain genes (Lawrence *et al.*, 2004), or by direct injection of specific growth factors (Faktorovich *et al.*, 1990) has also proven to be effective in delaying photoreceptor loss in the RCS rat.

8.1.3 Transplantation of photoreceptors and neural progenitors

Unlike RPE cell transplantation, that has been shown to promote photoreceptor survival and diminish further degeneration, the transplantation of actual photoreceptors or neural progenitors offers the possibility of a type of cellular therapy that would help those patients who have already lost their vision, providing that their inner retina remains intact (Fauser, 2007). Attempts have been made to transplant early postnatal mouse retinal photoreceptor cells into the subretinal space of the receptorless (rd) mutant mouse and normal mouse, resulting in the development and survival of transplanted photoreceptors for extended periods (Gouras *et al.*, 1994). The transplantation of human neural progenitors has shown promise, in that RCS rats receiving grafts (compared to treated rats) have retained near-normal acuity (Gamm *et al.*, 2007). The use of human embryonic stem cells in transplantation experiments with the RCS rat has also shown

promise in rescuing visual function, with no cells undergoing malignant transformation or overt immune rejection by the host retina (Lund *et al.*, 2006). In any cell transplantation strategy an inevitable hurdle that needs to be overcome is the possibility of immunorejection. By transplanting cells into the subretinal space this may inadvertently compromise the partial immune-privileged status of the region by disruption of the blood retinal barrier.

8.2 Experimental design and objectives

Transplantation of neuronal progenitor cells holds considerable potential for cellular therapy in degenerative eye diseases. In order to assess the potential of the unimmortalised and immortalised human foetal retinal progenitor cells in retinal cellular replacement, it is vital to observe their potential to integrate and express key antigens in a developing and diseased environment *in vivo*. It is hypothesised that this environment would be conducive towards driving differentiation of the retinal progenitor cells towards retinal neurons, and possibly rod photoreceptors.

Cells were trypsinized and resuspended in DMEM:F12 at 0.5 x 10^5 cells/μl (figure 8.1) of which 2 μl was injected into the RCS rats or 1 μl into the neonatal rats. All animals were anaesthetised by intraperitoneal injection, and after opening the eyelid cells or carrier medium were injected transclerally into the vitreous or subretinal space using a fine glass capillary pipette attached to a Hamilton syringe. All host animals (sham and cell grafted) were immune-suppressed two days prior to transplantation until being killed. At 5 days, 26 days and 9 weeks post grafting, rats were deeply anaesthetised and perfused intracardially, and eyes were removed and processed for immunohistochemical analysis of cryostat sections.

All grafting of animals in this chapter was performed by Dr. A. Vugler. Staining and imaging was carried out in collaboration, with some staining, including ED1, being carried out solely by Dr. A. Vugler.

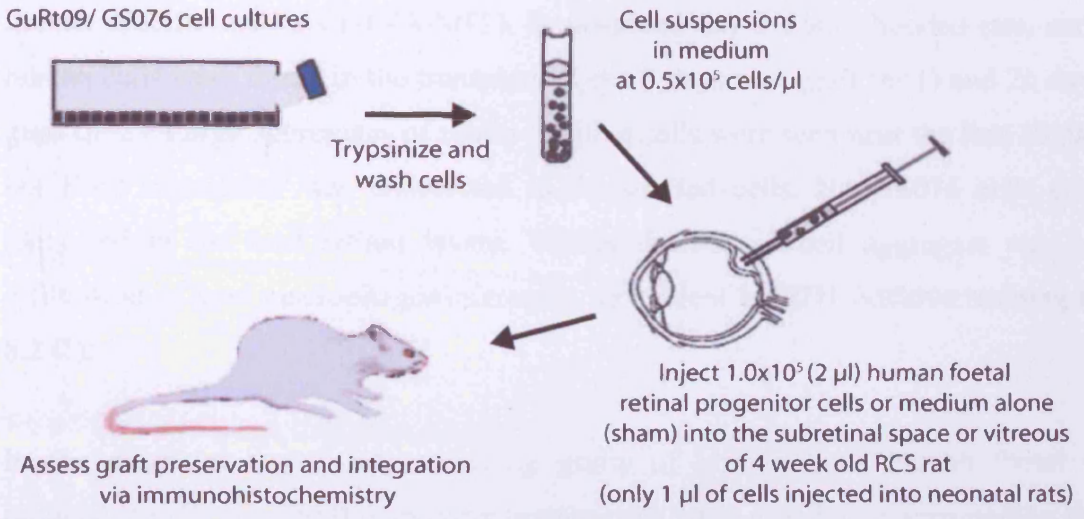

Figure 8.1 Diagrammatic representation of the transplantation procedure
Cells were grown in culture medium, trypsinized from the flask, and resuspended in DMEM:F12 medium. The cell suspension was injected into either the subretinal space or the vitreous using fine diameter glass capillary tubing attached to a 10 µl Hamilton syringe. Animals were placed on immunosuppression, and sacrificed 5 days, 3 weeks or 9 weeks post graft. After subsequent processing grafted cells were located via immunohistochemistry to assess cell preservation and integration. (Adapted from Lund *et al.*, 2003)

Postnatal day 3 Lister hooded rats (GS076 n=10 and GuRt09 n=10) all received intravitreal grafts, RCS dystrophic and non-dystrophic (8 weeks) rats received grafts of unimmortalised cells (n=12) and immortalised GuRt09 cells (n=8). Four week old dystrophic rats received unimmortalised and immortalised cells intravitreally and subretinally (n =12 of each). Transplanted human cells were identified in host eyes using the human specific markers, human nuclear antigen and human mitochondrial marker (HNA-MIT). Host macrophages and microglia were identified using the ED1 antigen that stains rat CD68 positive cells.

8.3 Results

Immunohistochemical examination of sectioned eye tissue revealed transplanted unimmortalised human foetal retinal progenitor cells (GS076) in the host eyes using human specific markers (HNA-MIT). In postnatal day 3 Lister hooded rats, surviving human cells were found in the transplanted eye 3 days post graft (n=1) and 26 days post graft (n=2). Large aggregates of nestin-positive cells were seen near the lens (figure 8.2), but Ki67 expression was undetected in the grafted cells. No GS076 cells could be identified in the host retinal layers. Within the human cell aggregate was a large infiltration of host macrophages/microglia, as evident by ED1 positive staining (figure 8.2 C).

In the postnatal day 3 rats receiving grafts of immortalised human foetal retinal progenitor cells (GuRt09), surviving human cells were also found surrounding the lens (figure 8.3). There was no evidence of retinal integration within the host eye at any of the time points assayed.

When unimmortalised cells were transplanted into vitreous of 8 week old RCS dystrophic rats (n=12) there was no evidence of retinal integration 5 days post graft (figure 8.4 A, n=1) or 26 days post graft (figure 8.4 B-G, n=2). However, there was a great deal of macrophage/microglia infiltration surrounding and migrating into the bolus of human cells (figure 8.4 A) as early as 5 days post graft. Even at 26 days post graft ED1 positive cells were clearly evident within the graft, suggesting possible immune rejection of the grafted cells. However, cells were nestin positive and a minority of cells expressed Ki67 (figure 8.4 B and C).

When unimmortalised cells were grafted into the subretinal space of the RCS dystrophic rat, 5 days post graft (n=3) and 26 days post graft (n=1), there were numerous macrophages/microglia infiltrating the HNA-MIT positive human cells (figure 8.5). When the sections were analysed immunohistochemically, recoverin, NF200 and rhodopsin were all undetectable in the cells. Only nestin expression was evident in these cells in the diseased retinal environment.

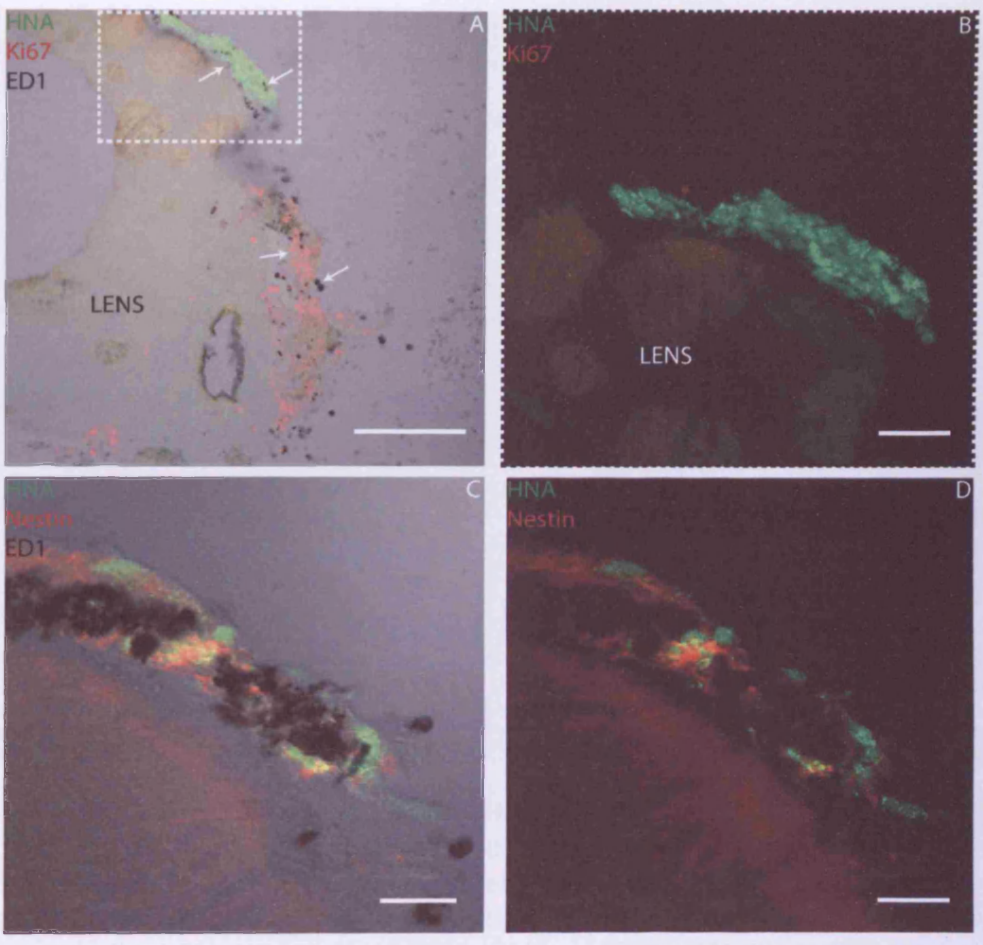

Figure 8.2 Expression patterns and location of unimmortalised cells transplanted into developing retina
Confocal images of retinal sections from neonatal rats (postnatal day 3) grafted with the unimmortalised human foetal retinal progenitor cells, GS076. Images taken 3 weeks post graft. (A and B) Cells, stained with human nuclear antigen marker (HNA) migrated towards the lens. The transplanted cells are postmitotic due to the lack of Ki67 expression. Ki67 expression is seen but from host cells. (C and D) Cells were also stained for Nestin, HNA and ED1. GS076 cells were only positive for nestin expression other antigens were undetectable. Scale bar= 300 µm (A) and 40 µm (B-D).

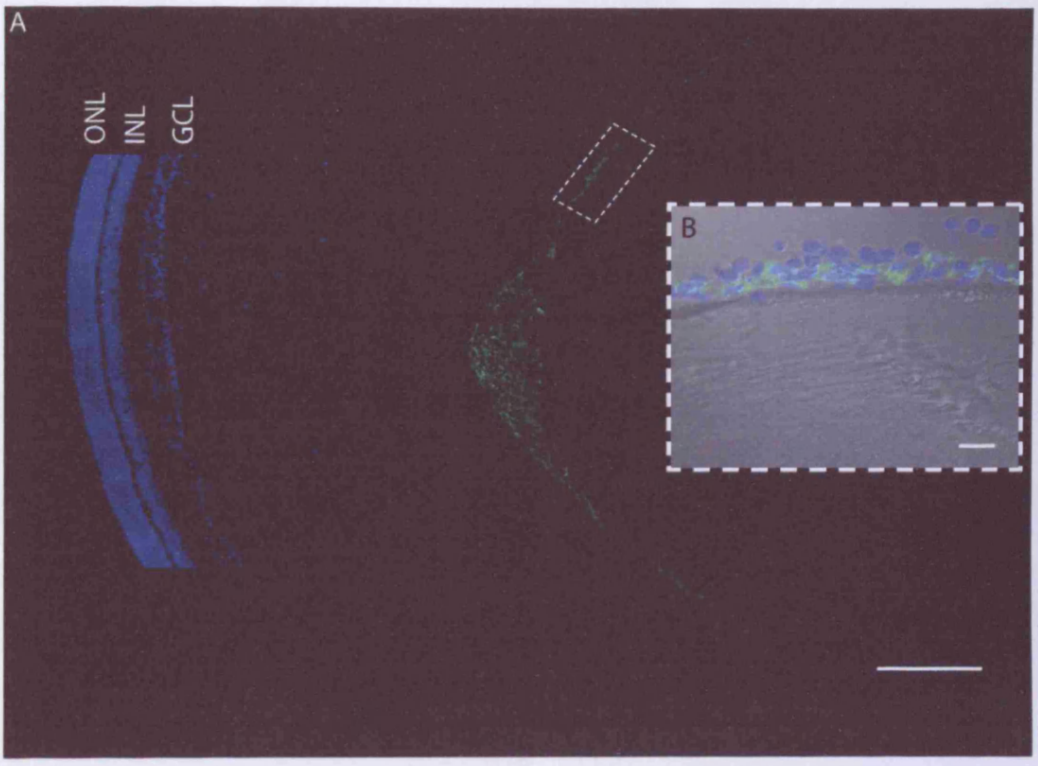

Figure 8.3 Location of GuRt09 cells grafted in neonatal (P3) rats 3 weeks post graft
Confocal images of the retinal sections stained with HNA-MIT marker to reveal transplanted cells of human origin. Cells were counterstained with DAPI (blue in all images) to reveal nuclei. There is no evidence of retinal integration as cells are found localised surrounding the lens. (B) magnified image of boxed section in A. Scale bar = 200 μm (A); 20 μm (B).

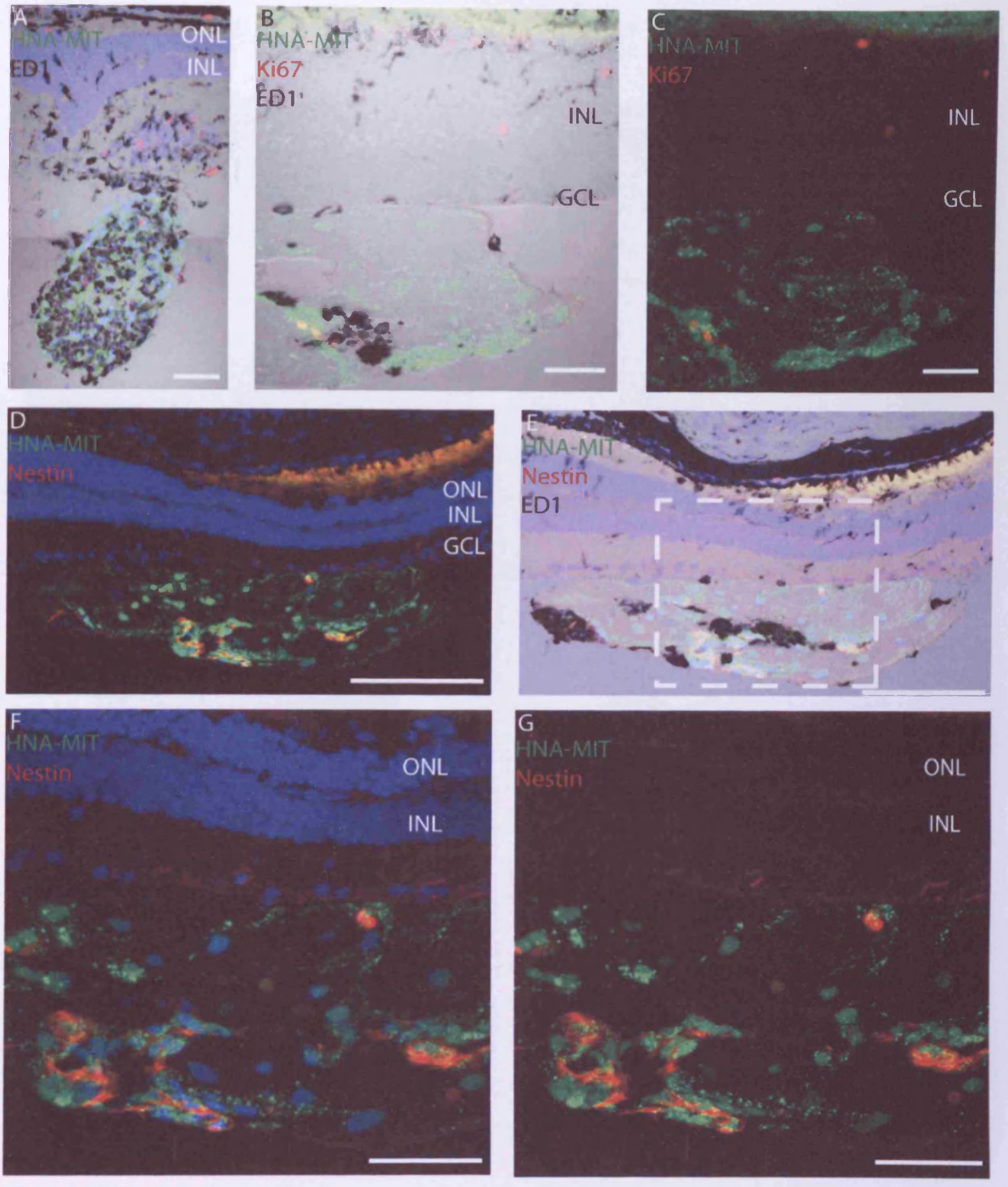

Figure 8.4 Unimmortalised cells transplanted into the vitreous of the RCS dystrophic rat
GS076 unimmortalised cells were grafted into the vitreous of 8 week old RCS dystrophic rats and analysed (A) 5 days post graft or (B-G) 26 days post-graft. Cells were stained with human mitochondrial and human nuclear antigen (HNA-MIT) Ki67, nestin and ED1 (specific for host CD68 found on macrophage and microglia. (A) Cells in the vitreous are surrounded by ED1 positive cells. (B,C) The majority of surviving cells, at 26 days post graft, were negative for Ki67 implying there exit from the cell cycle. However, microglia infiltration into the bolus of grafted cells was still present. (D) Cells were positive for nestin but microglia was present also (E). (F, G) Magnified image of the boxed region in (E) shows the HNA-MIT positive grafted cells also staining for the nestin anitgen at 26 weeks post grafting. ONL: Outer Nuclear Layer, INL: Inner Nuclear Layer, and GCL: Ganglion Cell Layer. Scale bar= 100 µm (A, F, G) 40 µm (B, C).

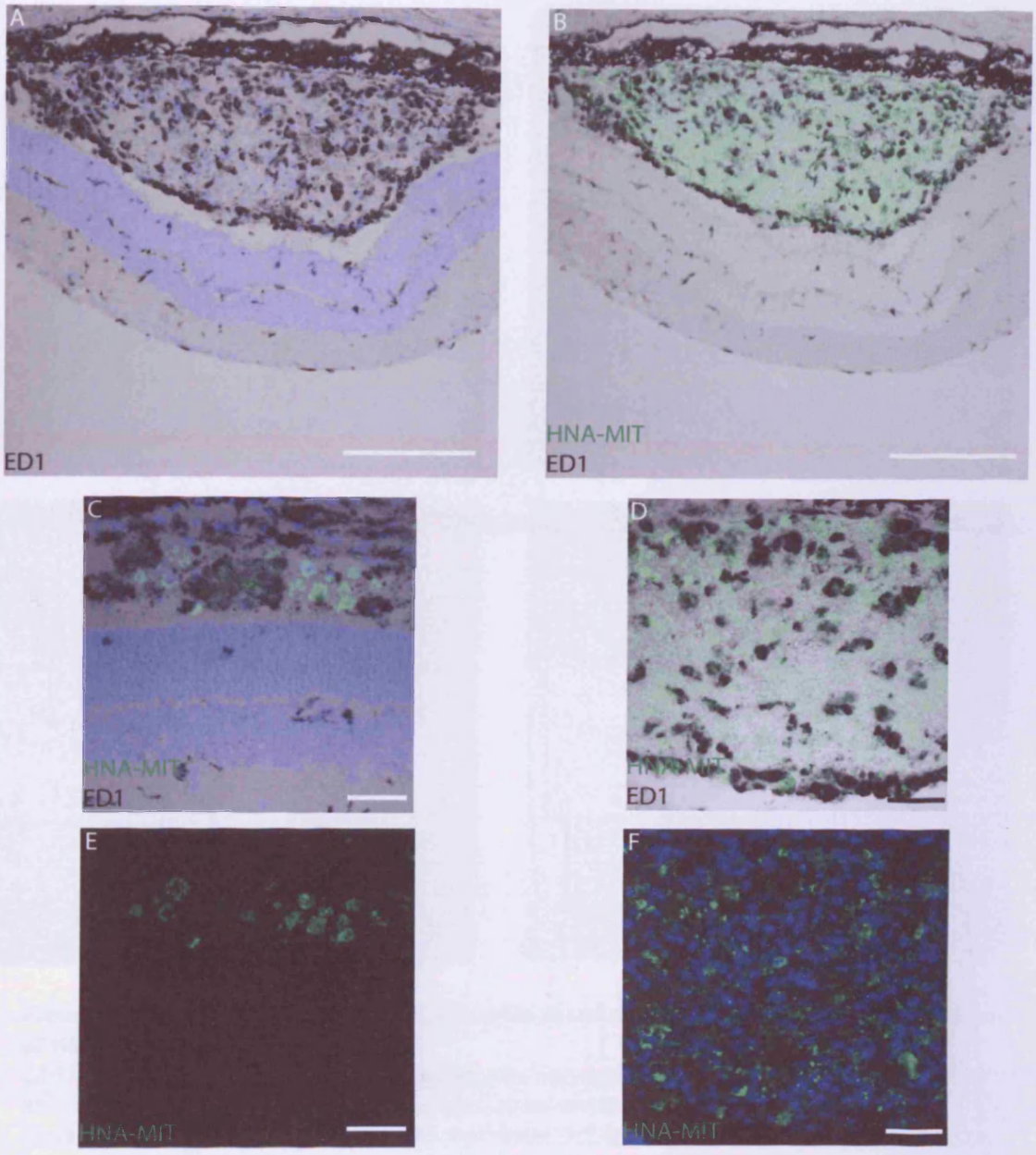

Figure 8.5 Unimmortalised cells transplanted into the subretinal space of RCS dystrophic rats
GS076 unimmortalised human foetal retinal progenitor cells were grafted into the subretinal space of a 4 week old RCS dystrophic rat retina. All images are representative of cells 5 days post graft (n=3). Nuclei were stained with DAPI (blue in images A, C and F). (A,B) Grafted cells stained with human nuclear antigen-mitochondrial marker are surrounded by ED1 positive cells in the subretinal space. (C,E) Magnified image of grafted cells in the subretinal space that are associated with ED1 positive cells. (D,F) Grafted cells within the subretinal space showing patchy staining of HNA-MIT and substantial macrophage infiltration. Scale Bar = 100 µm (A,B); 50 µm (C-F).

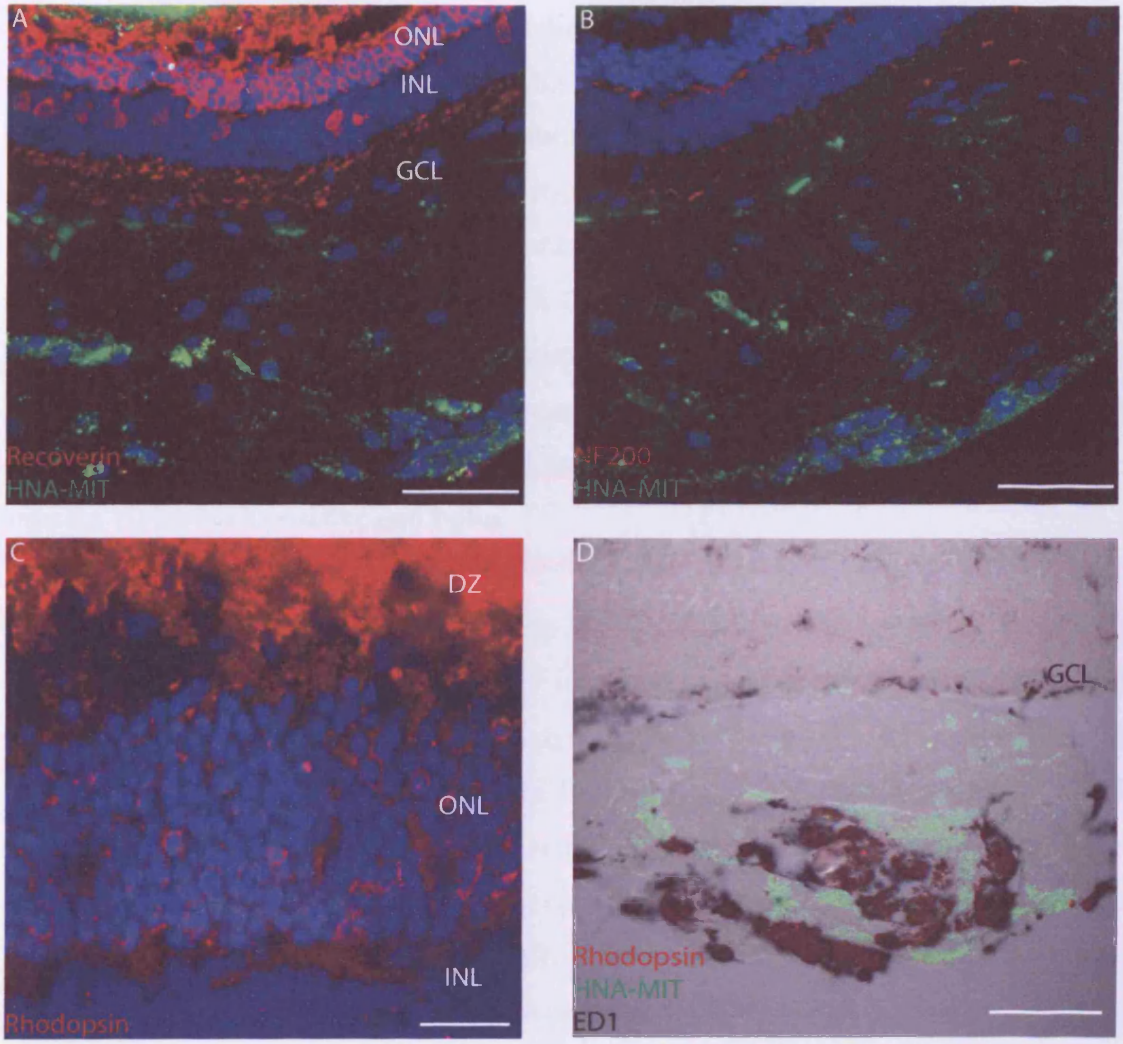

Figure 8.6 Expression patterns of unimmortalised cells transplanted into the vitreous of RCS dystrophic rats

GS076, unimmortalised cells transplanted into the vitreous of 8 week old RCS dystrophic rats and 26 days post graft were analysed immunohistochemically for the expression of various markers. (A) Recoverin expression was not detected in the grafted cells that were identified in host eyes by human specific antigens (human nuclear antigen and mitochondrial marker; HNA-MIT). (B) NF200 marker was not detected in the transplanted cells either. (C) Host retina expression of rhodopsin was localised to the outer nuclear layer (ONL) where the photoreceptor outer segments are located. (D) Host macrophage/microglia autofluorescence can lead to false results, the ED1 staining confirms that the apparent rhodopsin positive cells surrounding the HNA-MIT grafted cells are actually macrophage/microglia autofluorescence. All nuclei were stained with DAPI (blue in images A-C). Scale bar= 50 µm (A,B); 20 µm (C,D).

Immortalised human foetal progenitor cells transplanted into the subretinal space of 4 week old RCS dystrophic rats were analysed 5 days (n=4) post grafting. Sections examined at this time point failed to demonstrate any integration of grafted cells into the host retina. Here again there was evidence of macrophage/microglia infiltration with a plethora of ED1 positive cells surrounding the grafted cells. The immortalised cells behaved like unimmortalised cells in that they also expressed nestin. However, there were also differences, most notably immortalised cells still expressed Ki67 antigen (figure 8.8), though there was no detection of recoverin. S-opsin and rhodopsin were also analysed (figure 8.10) with no detection of S-opsin in the GuRt09 cells grafted into the subretinal space of 4 week old RCS dystrophic rats. Interestingly, with regards to rhodopsin expression, the bolus of human cells was embedded within rhodopsin-positive host cells, and what at first appeared to be rhodopsin expression in the GuRt09 cells, on closer inspection was resolved as host rhodopsin positive cells migrating deep into the HNA-MIT positive cell bolus.

Four week old RCS dystrophic rats were left for 9 weeks post-grafting at which point eyes were examined for the presence of unimmortalised and immortalised cells. In all eyes from the dystrophic (n=4 and non-dystrophic n=4) rats it was impossible to detect any unimmortalised cells. However, the immortalised cells were found to be present (figure 8.9) in 25% of the eyes of dystrophic rats and 50% of non-dystrophic rats examined, mostly at the interface between the vitreous and the retina. These cells still expressed nestin and were still actively within the cell cycle as evident by Ki67 expression. All transplantation results are summarised in table 8.1.

An unexpected result was the appearance of the unimmortalised cells in close association with collagen IV-expressing structures (figure 8.11) within the host eye. Collagen IV is an extracellular matrix protein and is a major constituent of Bruch's membrane. Collagen IV expression was also examined in the immortalised cell transplants, and interestingly, cells within the vitreous of 4 week old RCS dystrophic rats that were analysed 9 weeks post grafting, appeared to express collagen IV protein. It is therefore possible that the cells actually secrete their own extracellular matrix. Perhaps if the unimmortalised cells had survived 9 weeks post grafting, a similar pattern of expression of collagen IV would have been observed.

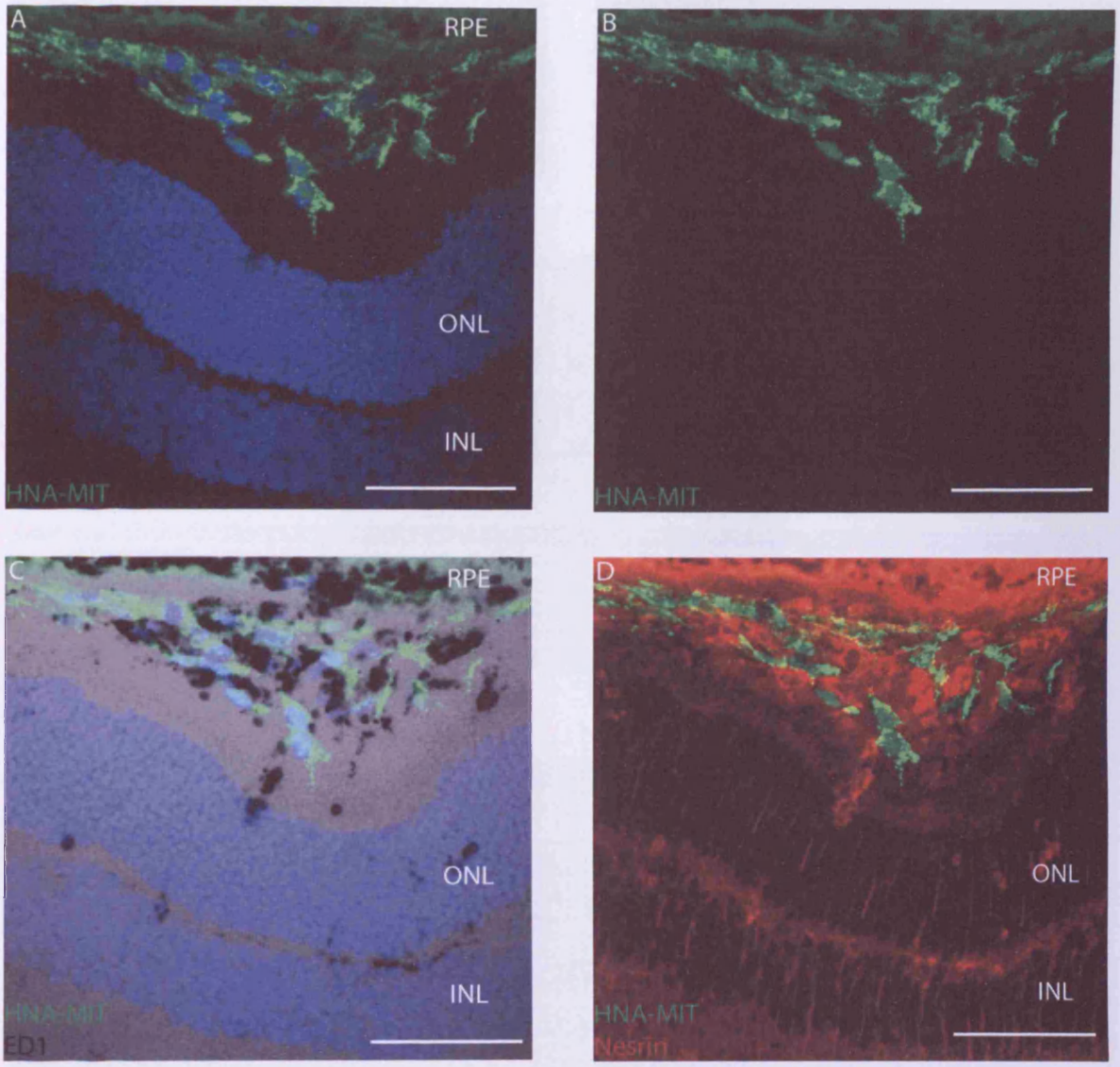

Figure 8.7 Immortalised retinal progenitor cells grafted into the subretinal space of RCS dystrophic rats
GuRt09 immortalised human foetal retinal progenitor cells were grafted into the subretinal space of 4 week old RCS dystrophic rats and analysed 5 days post grafting. (A, B) Cells were identified using a human nuclear antigen and mitochondrial marker (HNA-MIT) that showed cells isolated between the retinal pigment epithelium (RPE) cell layer and the outer nuclear layer (ONL). (C) However when host macrophage/microglia were examined, using the ED1 marker, there was an apparent host macrophage/ microglia response to the grafted human cells. (D) Grafted cells were apparently immuno-positive for nestin expression. All nuclei were stained with DAPI (blue in images). INL: Inner Nuclear Layer. Scale bar= 50 μm.

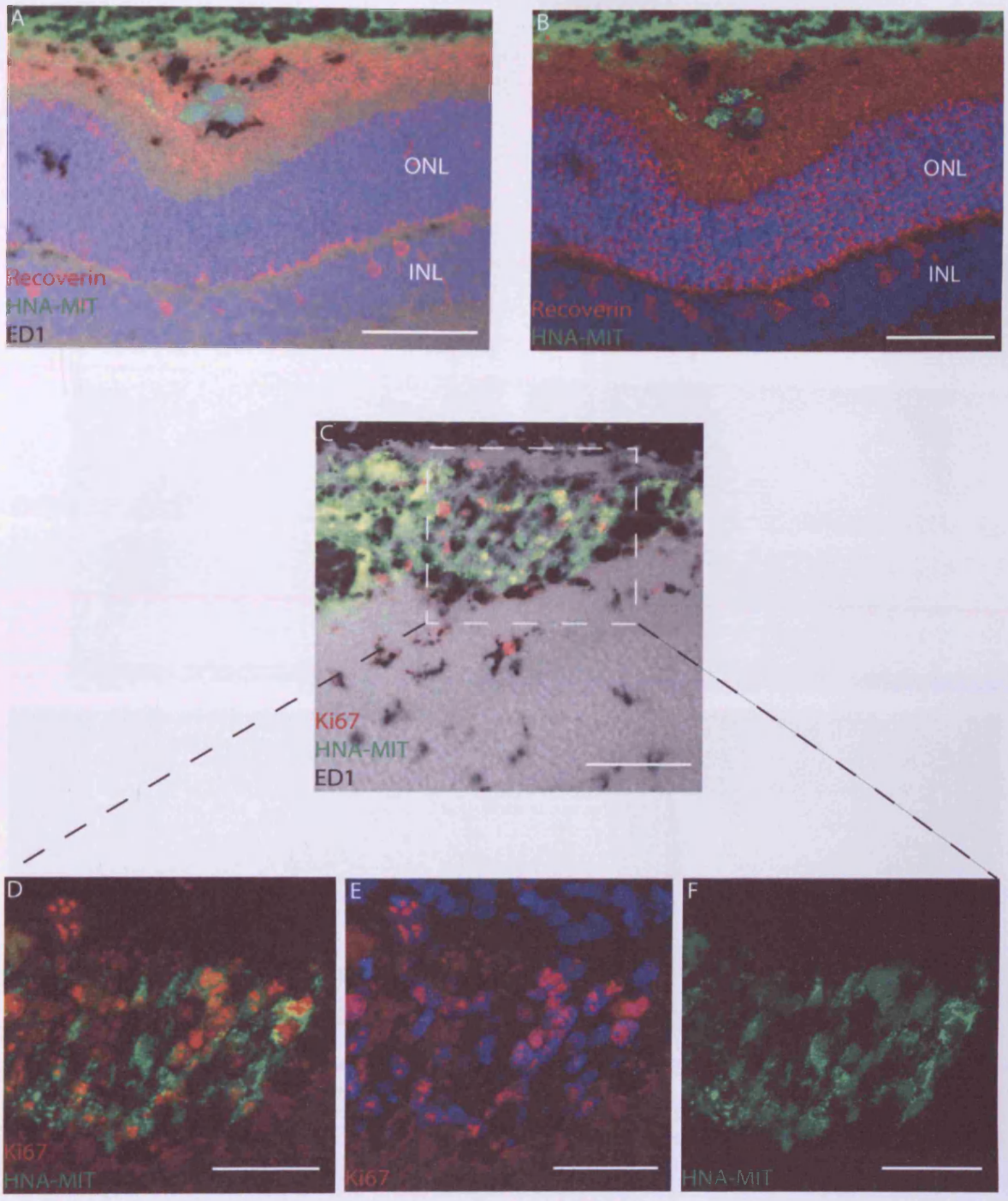

Figure 8.8 Expression profile of immortalised cells grafted into the subretinal space of the RCS dystrophic rat

GuRt09 cells were transplanted into the subretinal space of 4 week old RCS dystrophic rat retinae. Graft sites were analysed 5 days post grafting to observe the expression patterns of the immortalised cells *in vivo*. Cells were located using human specific antigens of human nuclear antigen and human mitochondrial marker (HNA-MIT) and further stained for various markers. (A, B) Cells were surrounded by recoverin positive cells from the host retina, however there was no detection of recoverin expression in the GuRt09 cells. (A) It is also apparent that ED1 positive host macrophage/microglia surround the grafted cells. (C) Transplanted cells were immunopositive for Ki67, found in all active parts of the cell cycle. (D,E,F) Magnified images of the boxed region in (C) showing the GuRt09 cells expressing Ki67 antigen. All nuclei were stained with DAPI, blue in images (A,B & E). Scale bar = 50 μm (A, B); 20 μm (C-F).

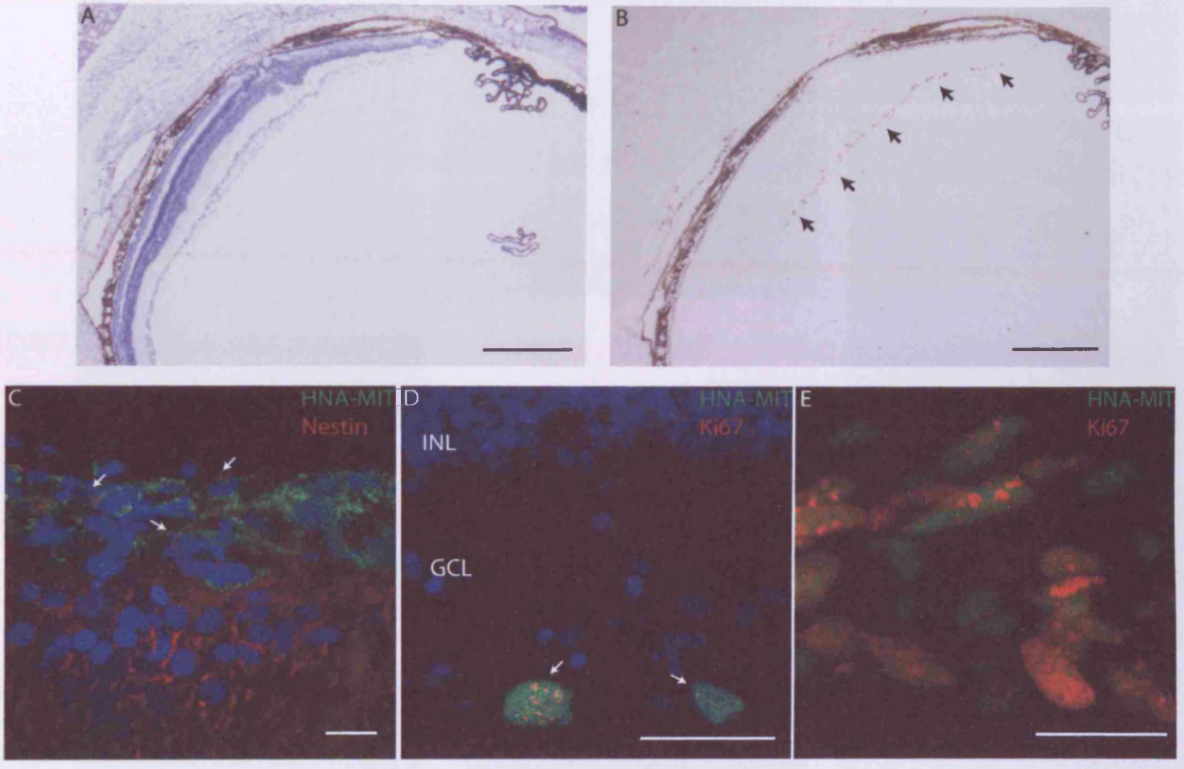

Figure 8.9 Immortalised retinal progenitor cells survive 9 weeks post transplantation in the RCS dystrophic rat
GuRt09 immortalised cells grafted into 4 week old RCS dystrophic rats survived *in vivo* for 9 weeks post graft. (B) Cells survived at the interface between the vitreous and retina as identified by the arrows. (C) Cells were stained for various markers, however the only markers that were detected were nestin and Ki67 (D & E). (C) Nestin was detected in the grafted human cells as indicated with the arrows. (D, E) Grafted cells within the vitreous were still actively within the cell cycle due to the presence of the Ki67 anitgen. INL: Inner Nuclear Layer, GCL: Ganglion Cell Layer. Scale bar = 600 μm (A,B); 20 μm (C, D, E).

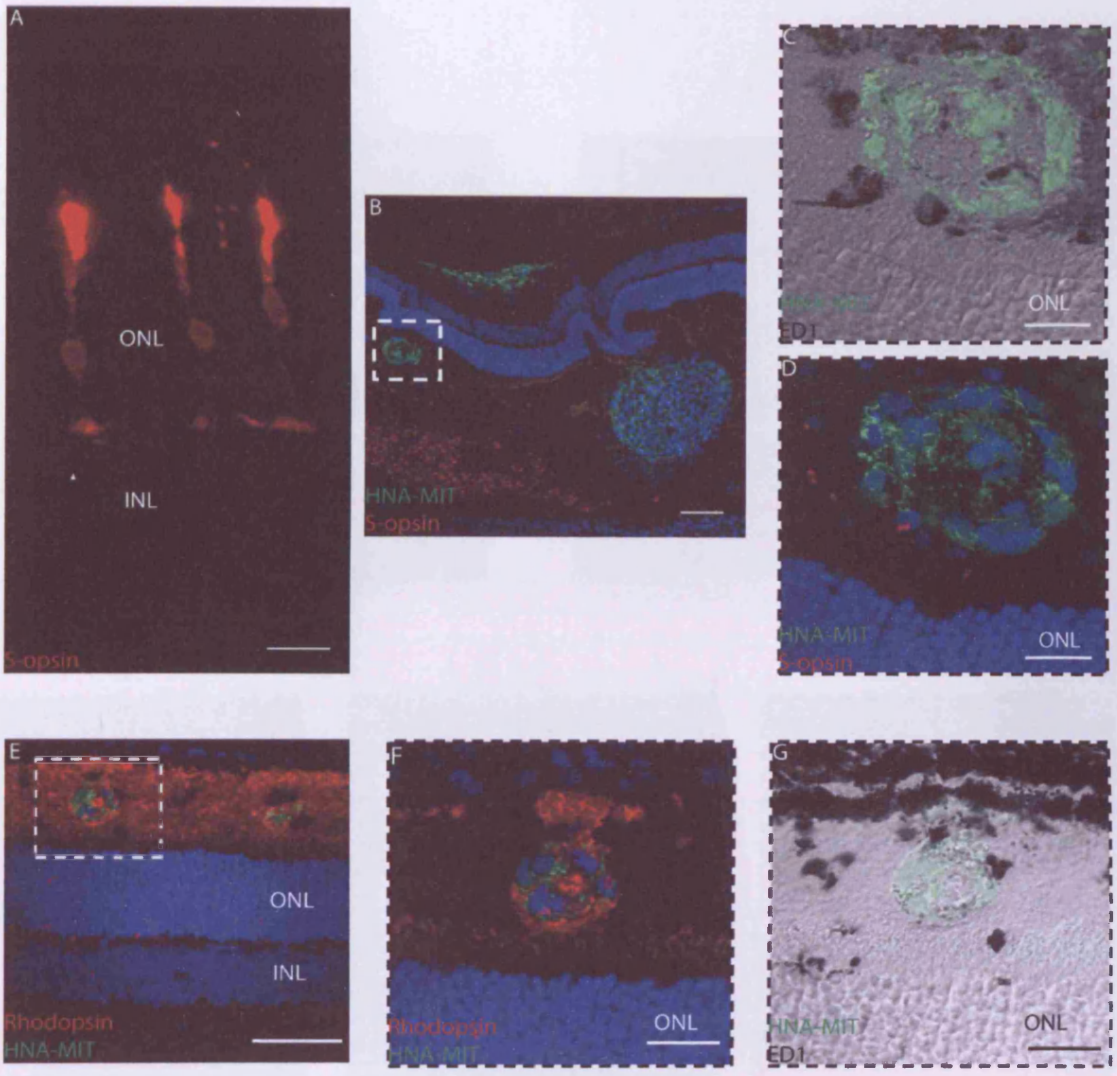

Figure 8.10 Expression of Opsins in immortalised cells grafted into the subretinal space of the RCS rat

GuRt09 immortalised cells were transplanted into the subretinal space of the 4 week old RCS dystrophic rat and analysed for the expression of rhodopsin and S-opsin 5 days post graft. (A) A section through a non-dystrophic rat retina showing outer segments of s-cone photoreceptors. (B) Immortalised cells formed bolus structures in the subretinal space but there was no detection of S-opsin expression in the GuRt09 cells *in vivo*. (C,D) Magnified image of the boxed region in (B) cells are surrounded by host macrophages/microglia. (E) Rhodopsin expression is clearly seen in the subretinal space where shed outer segments are present. The bolus of GuRt09 cells are within this region. (F,G) Magnified images of the boxed region in (E), the cells seem to be in close association with rhodopsin positive outer segments however the rhodopsin expression does not seem to be expressed by the transplanted cells themselves. Scale bar = 20 µm (A); 100 µm (B); 40µm (C, D) and 50 µm (E, F, G).

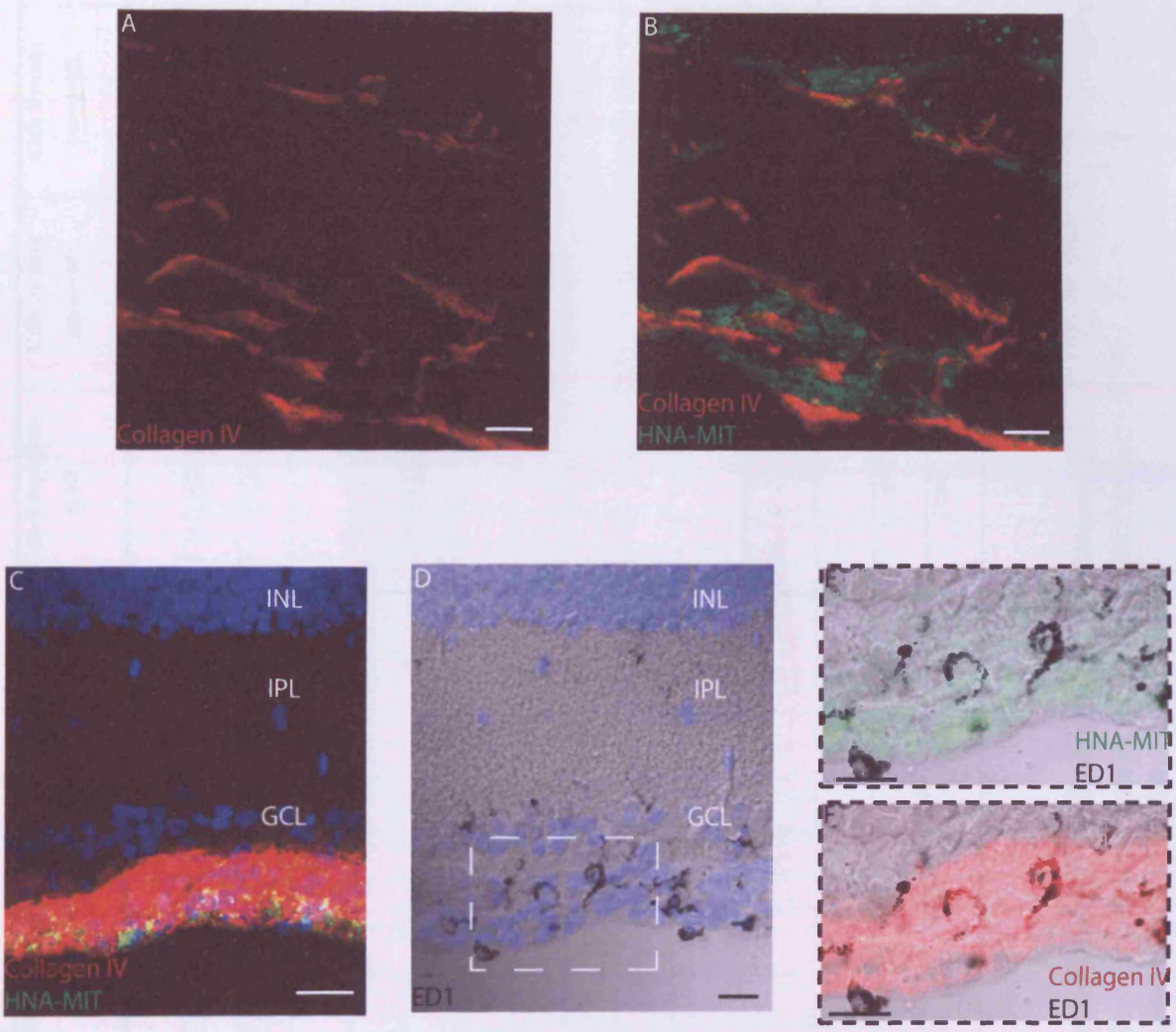

Figure 8.11 Collagen IV expression in transplanted human foetal retinal progenitor cells (A,B) GS076 unimmortalised cells were grafted into 4 week old RCS dystrophic rats and after 5 days post-graft cells were analysed. Interestingly, the cells stained with human nuclear antigen and human mitochondrial marker (HNA-MIT) became associated with collagen IV positive structures. Collagen IV antigen is an extracellular matrix protein. (C-F) GuRt09 immortalised progenitor cells were grafted into the vitreous of 4 week old RCS dystrophic rats and after 9 weeks post grafting the tissue was analysed, the GuRt09 cells seem to be expressing collagen IV. (E,F) magnified images of the boxed region in (D). All nuclei were stained with DAPI (blue in images). Scale bar= 40 µm.

Host	Age at grafting	Number grafted	Unimmortalised/ Immortalised	Cells 5 days post-graft	Cells 26 days post-graft	Cells 9 weeks post-graft	Graft location (SRS/vitreous)
Neonatal rat	Postnatal day 3	N=10	Unimmortalised	Yes (n=1)	Yes (n=2)		vitreous
Neonatal rat	Postnatal day 3	N=10	Immortalised	Yes (n=1)	Yes (n=2)		vitreous
RCS dystrophic	4 weeks	N=12	Unimmortalised	Yes (n=3)*	None		SRS
RCS dystrophic	4 weeks	N=12	Immortalised	Yes (n=4)	None		SRS
RCS dystrophic	4 weeks	N=4	Unimmortalised			None	vitreous
RCS non-dystrophic	4 weeks	N=4	Unimmortalised			None	vitreous
RCS dystrophic	4 weeks	N=4	Immortalised			Yes (n=1)	vitreous
RCS non-dystrophic	4 weeks	N=4	Immortalised			Yes (n=2)	vitreous
RCS dystrophic	8 weeks	N=12	Unimmortalised	Yes (n=1)	Yes (n=2)		vitreous
RCS dystrophic	8 weeks	N=8	Immortalised	None	Yes (n=1)		vitreous

Table 8.1 Summary of all transplantations undertaken using the unimmortalised GS076 cells and the immortalised GuRt09 cell line
Cells were either grafted into the vitreous or the subretinal region (SRS) of the host. Both cell types were present in the neonatal rat even though their localisation was confined to the lens region.

8.4 Discussion

In this chapter the developmental potential of unimmortalised and immortalised human foetal retinal progenitor cells has been evaluated *in vivo* in developing and disease environments. It has been demonstrated in previous chapters that these two cell types may express some retinal cell markers under certain *in vitro* conditions. Following transplantation, the phenotype of these human cells appeared to revert back to that of primary progenitor cells, with only the expression of nestin and in some cases Ki67 being observed. The cell cycle marker Ki67, was used to assess cells that had exited the cell cycle or were still actively within the cycle. A very low level of proliferation of unimmortalised transplanted cells were observed, however there was obvious Ki67 activity in the immortalised cells when grafted into the dystrophic rat retinae. This is consistent with the findings from the previous results chapters that while cells are actively with the cell cycle they are unlikely to be able to respond to the specific cues in order to commit and eventually integrate into the rodent retina. However, whilst the unimmortalised cells were Ki67 negative they did not show any degree of integration either. This could mean that the age of the cells has an impact on the degree of multipotency and differentiation and thus on the overall success of the graft (Armant *et al.*, 1988).

The differentiation status of retinal progenitor cells is an important determinant of their ability to integrate into the host retina. It is likely that induction of non-committed cells along specific lineages *in vitro* prior to engraftment, and modification of epigenetic signals in the host environment together with the grafting of the cells, is required to allow appropriate cell-specific differentiation and integration. However, as demonstrated in these experiments, induction of specific markers such as NF160, NF200, recoverin and S-opsin is lost when the cells are transplanted *in vivo*. This reveals the limited plasticity of the human foetal retinal progenitor cells, and also their apparent inability to respond to environmental cues.

This experimental strategy is further complicated by the appearance of host rejection, as judged by ED1 protein expression among the grafted cells. Original attempts at xenografting human neural progenitor cells (from foetal cortex) into rodent retina showed a failure of graft integration (Mizumoto *et al.*, 2001) which is consistent with the data here. Microglia play an important role in removing apoptotic neurons, and there is much evidence of increased numbers of activated microglia in animal models of

degeneration such as the RCS rat (Thanos and Richter, 1993). Microglia infiltration could therefore be a contributing factor in hindering the integration of the grafted cells. The infiltration of macrophages in a region believed to have some immune privilege may be due to the actual transplantation procedure itself, with the inevitable opening of the blood-retinal barrier (Lund *et al.*, 2003). The poor survival of grafted cells is consistent with findings elsewhere that the majority of transplanted neurons in CNS transplantation studies died by necrosis and apoptosis within 1 week of transplantation (Boonman and Isacson., 1999).

It is of great importance to avoid or control the potential immune or inflammatory responses that can occur as a response to transplantation. For several decades the eye, like the brain, has been known to possess a certain level of immune privilege that is not absolute, but which is relative to other organs. Immune privilege is not a passive process but is actively maintained (Streilein *et al.*, 1997), so that the eye can protect itself and its function from sight-threatening inflammation. The blood-retinal barrier, which is integral to the maintenance of immune privilege, is formed by an outer barrier in the retinal pigment epithelium and an inner barrier in the endothelial membrane, along with the junctional complexes, of the retinal blood vessels (Cunha-Vaz, 1979). The barrier regulates the microenvironment of the neuroretina and in doing so maintains the vitreous, retina and posterior segment of the eye as a 'privileged site' in the body (Cunha-Vaz, 2004). Alongside the blood-retinal barrier a host of components and secreted factors work together to deviate normal immune responses, including FasL (which functions to kill lymphoid cells invading the region), immunosuppressive cytokines, neuropeptides and a limited expression of MHC class I and II molecules all of which contribute to the immune privilege status of the eye (Ferguson and Griffith, 1997). Under normal conditions MHC molecules play a key role in the antigen-presenting process, however with few MHC molecules there are no true antigen presenting cells residing in the retina. This however can change when the blood retinal barrier is breached via the transcleral transplantation approach. This breach permits surveillance and access by antigen-presenting cells. Therefore wherever possible the breaking of the blood-retinal barrier should be avoided.

In conclusion, the xenografting model we described here has allowed us to assess the behaviour and functional capabilities of the human foetal progenitor cells. However, problems of graft rejection, failure of the graft cells to express photoreceptor markers,

and the absence of visible integration into the diseased or developing *in vivo* environment, suggest that significant obstacles remain if immortalised retinal progenitors are to be successfully developed as therapeutic tools.

Chapter 9
General discussion

Chapter 9

General discussion

The retinal dystrophies form an ever-expanding group of incurable diseases. With the prevalence of age-related macular degeneration (AMD) and retinitis pigmentosa (RP) in the developed world set to rise as a consequence of increasing longevity, new treatments for blinding eye diseases would be advantageous to public health and benefit the economy. Cell-based therapies offer the prospect of a viable and realistic method of treatment. The discovery of progenitor cells in the retina has driven forward research into the molecular cues required to direct these cells towards a particular lineage. However, the use of progenitor cells is finite due to the limited supplies and ethical implications in extracting such cell types. This creates a pressing need to generate cells for transplantation that would be safe, stable, and importantly, that can integrate, survive and differentiate to restore functional vision. Ideally, such cells must possess the characteristics of progenitor cells, but be expandable and easily generated. Immortalised progenitor cells could meet these objectives. The proliferative capacity of continuous cell lines would enable the preparation of bulk stocks, and permit extensive safety checks to be carried out prior to clinical trials.

The aim of work in this thesis was to characterise and evaluate the developmental potential of two clonally immortalised cell lines derived from the neural retina of 10-13 week old aborted foetuses. Studies were undertaken to determine their proliferative rate, expression of marker proteins and their ability to differentiate in various trophic conditions. The cells were also grafted into the retinae of neonatal hooded Lister, and diseased RCS rats, to assess survival, integration and differentiation. In some experiments, the cell lines were compared with the unimmortalised retinal progenitors of the same origin, in order to gauge the effects of immortalisation.

The cell lines were conditionally immortalised using an attenuated large T SV40 virus that allows the oncogene to be switched off at the non-permissive temperature of 37°C, as opposed to the permissive temperature of 33°C at which the cells actively proliferate. Conditional immortalisation is an important factor to consider if immortalised cells are to be used in clinical trials. It allows the expansion of cells under the influence of the large T antigen, however when transplanted, or grown at the non-permissive

temperature, the large T antigen should in theory be inactive. In effect, this creates two distinct populations of cells to investigate.

The SV40 tsA58 mutant used in this study has a single point mutation (Ala-438→Val) that renders it temperature sensitive (Reynisdóttir *et al.*, 1990). This mutant was used to immortalise the retinal cultures because it has been widely used, extensively studied and is regarded as the least "leaky" of the temperature sensitive mutants (Tegtmeyer and Ozer, 1971). When cells are infected, gene A from the SV40 genome is transcribed into A protein, also known as T antigen. It is this T antigen that is the immortalising oncogene (Cepko, 1989). The point mutation causes the T-antigen to undergo a conformational change at the non-permissive temperature and is believed to inactivate its immortalising ability (Truckenmiller *et al.*, 1997). The T antigen becomes defective in viral DNA replication at the non-permissive temperature, as it does not bind to the viral origin of replication therefore this leads to a decline in T antigen levels (Reynisdóttir *et al.*, 1990).

Initial comparisons with retinal progenitor cell cultures isolated at the same developmental stage indicated that the cell-lines shared certain expression patterns with the 'parent' cell type. All cell types expressed the neuroectodermal stem cell marker nestin, as well as Ki67, NF160, NF200 and recoverin. However only the unimmortalised cells expressed markers of later neuronal cell types, including ß III tubulin and S-opsin. Interestingly, RT-PCR analysis revealed that these two markers were present at the mRNA level in both cell lines. It would be of considerable interest to determine, at the mechanistic level, the nature of the molecular block that prevents translation of these mRNAs. If the block could be released, the cell lines may be more receptive to developmental cues. Although these data indicate that the unimmortalised cells may be more committed than the immortalised cell lines, it is important to keep in mind that the immortalisation procedure means that the GuRt09 and GuRt05 lines are individual clones, whereas the GS076 unimmortalised cells are composed of a heterogeneous population. This mixed cell population exhibited the variation in characteristics one would expect of a primary cell culture. Despite the observation that some cells within the primary culture may have been more committed or differentiated, the expression patterns between the cell lines and primary cells were broadly similar. This suggests that cultures of human retinal progenitor cells may indeed be transfected

with oncogenes, yet still maintain at least some of the characteristics of the primary cultures from which they were derived.

During vertebrate central nervous system development the interactions of progenitor cells with other cells and the microenvironment are essential. These interactions are especially difficult to maintain *in vitro,* when the environment is artificially engineered in order to encourage the development and eventual commitment of the cells towards a particular neuronal phenotype (Bronner-Fraser, 1992). However, studies in rat, mice and human retinal cell cultures have shown that an engineered environment can indeed induce the desired effects in the cells investigated (Gaur *et al.*, 1992; Angénieux *et al.*, 2006; Merhi-Soussi *et al.*, 2006; Sheedlo and Turner, 1996).

In this thesis several approaches were adopted to gauge the differentiation potential of the cell lines, including withdrawal of growth factors from the culture medium, addition of foetal bovine serum (FBS), addition of retinoic acid (RA) at varying concentrations, and the use of the ARPE19 cell line and its conditioned medium. With one exception (FBS), none of the above conditions induced any noticeable signs of differentiation in the GuRt09 and GuRt05 cell lines.

A major impediment in the all-*trans*-RA experiments was the apparent sensitivity of the cell lines to all-*trans*-RA, even at low concentrations. However, the ability of RA to induce cell death or growth arrest is well documented (Falasca *et al.*, 1998; Campochiaro *et al.*, 1991), and is consistent with the results observed here. In considering this issue, it is probably relevant that retinoic acid and its derivatives are used in anti-cancer treatments, as they inhibit cell proliferation and induce differentiation, growth arrest and apoptosis. Retinoic acid has been shown to induce growth arrest in specific tumour cells, including medulloblastomas, a type of human malignant brain tumour (Chang *et al.*, 2007). The mechanism by which this occurs has not been fully elucidated, although retinoid/receptor complexes are known to target genes involved in transcriptional regulation, including the proto-oncogene c-myc, and cyclin D1, both of which regulate cell cycle progression (Miller *et al.*, 1999). Chang *et al.,* (2007) found that after retinoid treatment of a medulloblastoma cell line, the expression levels of cyclin D1 and c-myc decreased, which resulted in cell cycle arrest. If these and very likely other oncogenes are affected by all-*trans*-RA, it is tempting to speculate that the SV40 large T antigen oncogene may respond in a similar manner.

However, this would still not answer the question of why the GuRt09 and GuRt05 cell lines were sensitive to RA at the non-permissive temperature. If the large T antigen is properly inactivated at 37°C, then the cells should theoretically lose the sensitivity observed. One possibility, discussed later, is that the mutant large T is not sufficiently regulated by temperature.

When the cells were cultured in all-*trans*-RA in the presence of 3% charcoal/dextran-treated FBS, there was a marked improvement in cell numbers. Thus, trophic factors supplied by the FBS appear to mitigate the potential problem of acute sensitivity to RA associated with transformation by the SV40 oncogene. The findings here contrast with other experiments (Kelley *et al.*, 1995), in which there was no observation in human foetal retinal cell cultures of diminished cell numbers, indeed with 500 nM all-*trans*-RA there was a significant increase in cell numbers and the expression of rhodopsin was observed. These discrepancies may be due to the fact that Kelley *et al* used primary cultures, thus comprising a heterogeneous population, within which different cells would have different responses to all-*trans*-RA, whilst the clonal GuRt09 and GuRt05 lines were more limited in this respect.

In considering these issues, we have touched on the possibility that the conditional immortalisation technique may not be as highly regulated and efficient in switching off tsT at the non-permissive temperature as would be necessary for cells to exit the cell cycle. This is generally viewed as being essential for differentiation, as retinal progenitor cells retain their multipotency until their final cell division (Marquardt and Gruss, 2002). If immortalisation using SV40 large T prevents cell cycle exit it would be extremely difficult to induce differentiation. However, it is essential to remember the nature of progenitor cells and their innate self-renewing and proliferative characteristics. An alternative hypothesis is that the trophic factors used throughout out this study were not capable of inducing differentiation. But since EGF, bFGF and RA and other agonists have been all been reported to induce retinal progenitor cell differentiation elsewhere, and in a variety of species, this seems unlikely.

With respect to the ARPE19 conditioned medium (CM) experiments, the results showed no significant up-regulation of any of the neuronal markers investigated, and neither was there induction of transcription factors or later cell markers including S-opsin, rhodopsin and Nrl. However, there was a significant elevation in Ki67 expression in

20% CM treated cells compared to the control cells. This implies that more cells are actively dividing when cultured in 20% CM than in the control cells. Many factors have been shown to be secreted by cultured retinal pigment epithelium (RPE) cells including bFGF and EGF (Bost *et al.*, 1992). bFGF and to a lesser extent EGF have been shown to affect differentiation of photoreceptors *in vitro* (Hicks and Courtois, 1992b), and to favour survival and proliferation of cells respectively. In experiments undertaken in Chapter 4, we observed an increased number of cells over a duration of 7 days in cultures treated with bFGF and EGF, compared to the cells in control medium. However, statistical analysis did not show a significant difference in Ki67 expression between treated and control cultures. There was also no difference in expression patterns of several retinal markers between the treated and control cells when exposed to growth factors. In comparison, a similar study observed photoreceptor generation, with rod and cone specific antibodies, when immortalised human retinal precursor cells were treated in the presence of bFGF or EGF. These results raise the possibility that even with the presence of SV40 large T antigen activated (this was not a conditionally immortalised cell line) bFGF and EGF could have an inductive effect on photoreceptor generation whilst cells are in the cell cycle (Ezeonu *et al.*, 2000).

These data, when taken together with the ARPE19 CM experiments, reveal that bFGF and EGF do have a proliferative effect on retinal progenitor cells, and that in combination with other trophic factors released from the ARPE19 cells, this may enhance the number of dividing cells. However, these data also demonstrate the inability of the immortalised human foetal retinal progenitor cells to exit the cell cycle at the non-permissive temperature of 37°C, which as mentioned earlier probably contributes to the lack of differentiation, or may simply reflect a developmental stage at which the cells have lost their progenitor cell characteristics.

The developmental potential of these immortalised retinal progenitors may therefore be somewhat limited. The only trophic factor that had a discernable effect on differentiation was foetal bovine serum (FBS), which activated the translation of the ß III tubulin gene, a ganglion cell marker, in addition to the expression of NF160 and NF200 neurofilament proteins that are expressed in the axons of ganglion cells (Kivelä *et al.*, 1986). This shows the potential of these cells to differentiate, even though the sustained expression of nestin suggests that the cells retain certain 'stem-like' characteristics. In a temporal sense, the development of neuronal retinal cell types

begins with the emergence of ganglion cells, some markers of which were observed in the immortalised cell lines. A possible next step would be to culture the cells in FBS, and then to switch them into medium containing ARPE19 CM. As demonstrated by Lamba and colleagues (2006), the key to obtaining maximal retinal lineage commitment from human embryonic stem cells involves identifying the correct combinations of molecular cues to optimize efficiency of target cell derivation. In this way sequential exposure of the cells to different factors over weeks or months would make sense, as the development of rhodopsin positive cells occurs slowly *in vivo.* Perhaps cells cultured *in vitro* need just as long to adapt and respond to the developmental cues.

An approach that is well established at the Institute of Ophthalmology for studying tissues and cells in retinal differentiation is transplantation into developing or diseased ocular environments. When the immortalised GuRt09 cell line and GS076 retinal progenitor cells were transplanted into neonatal hooded Lister rats, both cell types migrated towards the lens region in the eye. As the surface ectoderm (presumptive lens) plays a role in neural retinal specification (Hyer *et al.*, 1998), it is not unexpected that the cells migrate to this region. Since the surface ectoderm is fully differentiated at the age the rats were used it is possible that the lens retains some vestigial ability to secrete factors that the developing progenitor cells might respond to, and that could be essential for their development. Unexpectedly, the cells did not integrate into the retina, which either implies that they are not competent to respond to the cues in the neural retina or that retinal membranes present a physical barrier to integration, or that the retina simply does not produce recruitment or attraction factors. In other studies integration and differentiation have been observed from human embryonic stem cells (Banin *et al.*,2006) and retinal progenitor cells (Chacko *et al.*, 2000).

To address this problem, cells were transplanted subretinally into RCS dystrophic rats, though again there was no visible integration into the neural retina. Interestingly, in the retinal environment, the cells seemed to revert to a more multipotent state, with only the expression of nestin and Ki67 apparent. The expression of Ki67 in the immortalised cells at 37°C further indicates that the temperature-sensitive immortalisation may not be tightly regulated. However, this is not the case in every SV40 conditionally immortalised neural progenitor cell line. Embryonic rat striatal-derived neuronal progenitors that have been transplanted into rat foetuses have shown an inactivation of SV40 T-antigen between 6 and 24 hours after transplantation. This is coupled with a

progressive reduction of mitotic activity of the immortalised cells (Cattaneo *et al.*, 1994). This suggests that although the conditional immortalisation in this study may not have been tightly regulated, it has been shown to be effective in other studies. A further possibility is that the SV40 retrovirus integrated into the genomic DNA in a critical region for the regulation of cell proliferation.

Microglia play an important role in removing apoptotic neurons, and there is much evidence of increased numbers of activated microglia in animal models of degeneration such as the RCS rat (Thanos and Richter, 1993). Abundant microglia were also apparent in the transplants performed here, that seemed to engulf and surround the bolus of grafted cells. It is possible that pre-treatment of either or both cell types with the trophic factors discussed earlier, may alleviate this problem by modulating their behaviour following transplantation. Especially with regards to the FBS-treated cells, it would have been interesting to observe whether partial differentiation of the immortalised human foetal cells *in vitro* might enhance integration *in vivo*.

A consistent feature throughout this study was that the immortalised cells always expressed Ki67, even *in vivo* following transplantation. This inability to become post-mitotic is most likely the result of a weak conditional immortalisation control, which contributes to residual cell proliferation at what should be the non-permissive temperature (May *et al.*, 2005). For clinical use, a method of conditional immortalisation that is tightly regulated and which can be unequivocally switched off, would be essential for reasons of safety, and would allow greater control of proliferation *in vitro*. In contrast to the results observed here, the ability of conditionally immortalised neural cell lines to accurately perform their *in vivo* functions has been demonstrated in studies elsewhere. Neural rat progenitors, taken from the embryonic hippocampus and striatum, differentiate into mature glia after transplantation into the adult rat brain with full integration into host cell populations (Lundberg *et al.*, 1996). A possible reason the cells did not integrate in the RCS rat retinae may be due to the finding that immortalised transplanted cells tended to 'avoid' areas of lesion or damage (Shihabuddin *et al.*, 1996), however this study did find differentiation and integration of the RN33B cell line when transplanted into intact dentate gyrus. This suggests that this cell line may require direct cell-cell interactions with intact neurons to differentiate. In contrast, it has been found that immortalised RPE cells respond well in a degenerating environment, and have shown preservation of visual function in the RCS rat (Coffey *et*

al., 2001). This still does not explain why the cells transplanted into intact neonatal retinae did not migrate towards or integrate within the retina.

Another aspect to consider is the stage at which the cells are immortalised. As each cell line presumably originates from a single cell within a heterogeneous population, it is possible that the original transfected cell was already partially committed. As the cells can be induced to express ß III tubulin, this shows that they have the ability to begin to differentiate in the sequential manner expected of a progenitor cell. But it could be that they were already partially committed at the time of immortalisation to differentiate along this lineage, although other observations suggest this may not be the case. For example, the detection of S-opsin at the mRNA level reveals they still possess some multipotency. Other human foetal cells studied to date have shown a high degree of plasticity, and the ability to differentiate (Kelley *et al.*, 1995; Dutt *et al.*, 1994), which to some extent was also evident here with the immortalised human foetal retinal progenitor cells.

In conclusion, the methods adopted in this thesis have shown that conditionally immortalised human foetal retinal progenitor cells retain a limited capacity to differentiate *in vitro*. This provides a basis from which to further investigate the possibility of using immortalised cells in a clinical setting. The real problem in this study was the inability of the cells to exit the cell cycle, which prevented them from becoming post-mitotic, and responding to environmental cues that have been proven elsewhere to induce differentiation. This problem may not have arisen with a transcriptionally regulated immortalisation technique, and future studies would ideally utilise immortalised cells generated in this way. In parallel, it would be important to undertake a closer examination of foetal progenitor cells in their primary state. Even though this has proven difficult in this thesis it is important to gain as much information as possible from progenitor cells in order to understand how best to manipulate them for future experiments and eventually for cellular therapy.

The aim is to build upon the studies presented in this thesis to provide a treatment for patients with retinal disease. Therefore it is important to evaluate what more could have been done to progress the field further.

A key area that would benefit from greater exploration is the immortalisation technique used in this study. The SV40 tsA58 temperature sensitive (ts) mutant is regarded as the least "leaky" of the ts mutants, however some studies suggest the propensity of the large T antigen to remain partially active even after incubation at the non-permissive temperature, requiring anywhere from 10-20 minutes in order to become fully heat inactivated (Reynisdottir *et al.*, 1990). Therefore the switch is not absolute but a gradual process. Another aspect that may warrant further investigation is the non-permissive temperature the retinal progenitor cells are placed at. In other studies it has been found that the higher temperatures of 41°C have shown total inactivation of the large T antigen.

It would also be advisable to sequence the DNA for the large T antigen (gene A) from each retinal progenitor cell line, in order to identify the tsA58 point mutation and ensure that it was present and that no other aberrant mutations had occurred. In a similar study, DNA sequence analysis showed that a cell line immortalised with the tsA58 mutation had reverted to the wild type T-antigen (Truckenmiller *et al.*, 1997). Although reversion to the wild-type may be relatively rare, this could account for the continued proliferation of the GuRt09 and GuRt05 cell lines at the non-permissive temperature.

It has previously been mentioned that the use of a transcriptionally regulated conditional immortalisation method as opposed to a temperature-sensitive method may provide a more definitive switch. A regulatable immortalising transgene suitable for this purpose is c-mycER. The use of a fusion protein composed of an inactive mutant of the oestrogen receptor fused to the c-terminus of the c-myc protein could be more tightly controlled (Littlewood *et al.*, 1995). The use of a transcriptionally inactive mutant of the oestrogen receptor is vital as it is unable to bind to oestrogen, therefore any endogenous oestrogen present will not interfere with the activity of the fusion protein. The proto-oncogene's expression is therefore activated with a synthetic ligand of 4 hydroxy-tamoxifen (OHT) instead. When OHT is present in the medium, the transfected cells will activate c-myc expression which in turn maintains the cells within the cell cycle, which is comparable to the SV40 large T antigen at the permissive temperature. Therefore, when cells are required to stop dividing and exit the cell cycle, OHT treatment is stopped allowing c-myc to be switched off. Perhaps this method of immortalisation would have been more tightly regulated, and would have enabled us to

detect more significant differences in gene expression patterns in the two cell populations.

With regards to the ARPE19 conditioned medium (CM) experiments, rather than simply collecting the CM from the ARPE19 cells, in future work it might be more appropriate to co-culture the ARPE19 cells in transwell plates with the immortalised retinal progenitor cell lines. Previous studies have mentioned that co-culturing is pivotal for progenitor cells to become differentiated (Liu *et al.*, 1988). Therefore, if there were trophic factors present in the CM collected that were only viable over a short distance and/or had a short functional half-life there would be a higher probability that these factors would come into contact with the retinal progenitors in a co-culture set-up and perhaps exert some detectable influence.

A diffusible factor identified in RPE conditioned medium and known to have a potent effect on neuronal differentiation is Pigment Epithelium-Derived Factor (PEDF) (Tombran-Tink and Johnson, 1991). PEDF is now available commercially, therefore it would be interesting to investigate the effects, if any, of this growth factor on the immortalised retinal progenitor cell lines. Even though this was one of the factors we expected to be secreted from the ARPE19 culture it may not have been present, or may have been secreted at levels too low to show an effect. An investigation of PEDF could help us gain more information on the mechanisms involved in retinal differentiation. Alongside the use of PEDF, the use of different RPE cell lines may yield distinct responses in the retinal progenitor cells. Even though ARPE19 cells share some traits with their parental cells they also differ in several key aspects. Ideally, any future study should include experiments that utilise primary RPE cultures instead of cell lines.

It would also be of great interest to investigate the effects of co-culturing the immortalised retinal progenitor cell lines with early human retinal tissue. As the explant would have been in the process of development, it is reasonable to assume that a larger and perhaps more beneficial set of trophic factors would have been secreted, with a more direct role in mediating the differentiation of the cell lines. Several other studies have shown that co-culturing progenitor or stem cells with either developing retina or retina from a mouse model of retinal degeneration promotes photoreceptor differentiation (Ikeda *et al.*, 2005; Lamba *et al.*, 2006).

If more time had been available I would have liked to repeat the bFGF and EGF experiments but investigate the effects of both mitogenic peptides separately in order to observe if either one had a greater effect on proliferation or differentiation alone. With regards to bFGF there have been reports that this peptide plays a role in photoreceptor development (Hicks and Courtois, 1992b). However, the younger the cells the more effective the bFGF seems to be in inducing rhodopsin expression. If the unimmortalised retinal progenitor cultures were easier to maintain in culture it would have been of great interest to see if these primary cells would have responded differently to the immortalised retinal progenitor cell lines when placed under the same culture conditions.

When cells are taken from late embryonic to early postnatal retina, higher concentrations of the mitogens are required, than when compared to early embryonic cultures, where maximal effects are observed with the lowest of mitogen concentrations (Lillien and Cepko, 1992). This seems to imply that foetal cells that are developmentally mid-way between these time points may require higher concentrations of growth factors to elicit maximal effects. However, the experiments were carried out at concentrations for both bFGF and EGF reported to be effective in previous studies (Hicks and Courtois, 1992b). The possibility remains that with higher concentrations of both growth factors, in combination or separately, the treated cultures could show higher proliferation rates or signs of differentiation. This would be another area to investigate further.

Throughout this study the key aims have been to establish a cocktail of trophic factors in the cellular microenvironment that mimic the *in vivo* developing environment. In the longer term it should be possible to build on the results obtained in this thesis, which do show at least some potential of these immortalised human foetal retinal progenitor cells to differentiate *in vitro*, and advance the application of retinal progenitor cells as a therapeutic option for retinal degeneration.

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